ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY

An International Journal

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SETAC

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ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY

Editor-in-Chief
C.H. Ward
Rice University

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AIMS AND SCOPE

Environmental Toxicology and Chemistry, the official journal of the Society of Environmental Toxicology and Chemistry (SETAC), is dedicated to furthering scientific knowledge and disseminating information on environmental toxicology and chemistry, including the application of these sciences to hazard/risk assessment. The journal provides a forum for professionals in academia, industry, government, and other segments of society involved in the use, protection, and management of the environment for the enhancement of ecological health and human welfare.

Environmental Toxicology and Chemistry is divided into three sections, each with its own editors: environmental chemistry, environmental toxicology, and hazard/risk assessment. Interdisciplinary in scope, the journal includes integrative studies involving components of classical toxicology; physiology; biology; microbiology; organic, environmental and analytical chemistry; anatomy; genetics; environmental engineering; geology; ecology; soil, water and atmospheric sciences; and economics.

Through peer-reviewed research papers, short communications, and review articles, *Environmental Toxicology and Chemistry* reports concepts and the results of experimental and analytical studies that can be used for the development of ecologically acceptable practices and principles.

ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY

An International Journal

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Editorial

SETAC

A PROFESSIONAL SOCIETY FOR ENVIRONMENTAL SCIENTISTS AND ENGINEERS AND RELATED DISCIPLINES CONCERNED WITH ENVIRONMENTAL QUALITY

The Society of Environmental Toxicology and Chemistry (SETAC), with offices currently in North America and Europe, is a nonprofit, professional society established to provide a forum for individuals and institutions engaged in the study of environmental problems, management and regulation of natural resources, education, research and development, and manufacturing and distribution.

Specific goals of the society:

- Promote research, education, and training in the environmental sciences.
- Promote the systematic application of all relevant scientific disciplines to the evaluation of chemical hazards.
- Participate in the scientific interpretation of issues concerned with hazard assessment and risk analysis.
- Support the development of ecologically acceptable practices and principles.
- Provide a forum (meetings and publications) for communication among professionals in government, business, academia, and in other segments of society involved in the use, protection, and management of our environment.

In pursuing these goals, SETAC and its members conduct numerous activities:

- Hold annual meetings composed of study and workshop sessions, platform and poster papers, and presentation of achievement and merit awards.
- Sponsor a monthly scientific journal, a newsletter, and special technical publications.
- Provide funds for education and training through the SETAC Scholarship/Fellowship Program.
- Organize and sponsor chapters to provide a forum for the presentation of scientific data and for the interchange and study of information about local concerns.
- Provide advice and counsel to technical and non-technical persons about scientific issues through a number of standing and ad hoc committees.

SETAC membership is currently composed of 3,600 individuals from government, academia, business, and public-interest groups with technical backgrounds in chemistry, toxicology, biology, ecology, atmospheric sciences, health sciences, earth sciences, and engineering.

If you have training in these or related disciplines and are engaged in the study, use, or management of environmental resources, SETAC can fulfill your professional affiliation needs.

Members—Must have applied experience, education in environmental sciences or engineering, toxicology or chemistry, or conducted research in areas related to the Society's stated goals:

- Doctorate plus two years experience
- Master's plus four years experience
- Bachelor's plus six years experience
- Special appointment by the Board of Directors

Associate Members—Must have training in and be engaged in lines of work related to the Society's purpose.

Student Members—Must be actively engaged in an academic curriculum leading toward a career in environmental sciences or engineering, toxicology, or chemistry.

Senior Active Members—Must have been SETAC members in good standing before changing their status, must be 62 years of age, and must not be fully employed. Senior Active Member dues are one-half of those of full/associate members. Senior Active Members do not receive the journal but have the option of purchasing the journal at the current rate.

Emeritus Members—Selected and so honored in recognition of their contribution to the Society and to environmental toxicology and chemistry or related professionals.

All members receive a newsletter highlighting environmental topics and SETAC activities, and reduced fees for the Annual Meeting and SETAC special publications. Members may hold office and, with the Emeritus Members, constitute the voting membership.

All members except Students and Senior Active Members receive monthly issues of *Environmental Toxicology and Chemistry* (*ET&C*), a peer-reviewed journal of the Society. Student and Senior Active Members may subscribe for $32 per year.

Please complete the accompanying application for membership, or if you desire further information, write or call the appropriate SETAC office.

SETAC	**APPLICATION FOR MEMBERSHIP**

SOCIETY OF ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY

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Circle Class of Membership:

	SETAC	SETAC-Europe
Member / Associate Member	$90 per year	70 ECU per year
Student Member (no journal)	$32 per year	20 ECU per year
Student Member, with journal	$64 per year	50 ECU per year

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THE BOARD OF DIRECTORS HAS REQUESTED THIS INFORMATION ON A VOLUNTEER BASIS.
THE INFORMATION IS FOR SETAC INTERNAL USE ONLY; DATA WILL NOT BE RELEASED WITHOUT BOARD APPROVAL.

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Membership in SETAC begins on January 1st, and membership applications received before October 1st will be for the current year. Successful applicants will receive all journals, newsletters, and other member benefits for the calendar year. Membership applications received on or after October 1st will apply to the next calendar year. In either case, applicants will be entitled to member registration for the Annual Meeting.

Student Status Confirmation: ALL STUDENTS MUST COMPLETE.

_____   _____
Faculty Advisor Signature   Date

_____
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YOU WILL BE CONTACTED AFTER YOUR APPLICATION HAS BEEN REVIEWED BY THE MEMBERSHIP COMMITTEE.

ETC 1996

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Editorial

LOOKING FORWARD: ENGAGING SETAC AND ITS SCIENCE MORE BROADLY

The founders of the Society of Environmental Toxicology and Chemistry (SETAC), with what time has proven to be 20/20 foresight, sought to establish a forum for effective, interactive communication among scientists in the emerging disciplines of environmental toxicology and environmental chemistry. The Society of Environmental Toxicology and Chemistry is fundamentally a society of applied science, as evidenced by the nature of those organizations represented by its founders and its present membership. Granted, basic, classic science is our foundation; this is clearly evident in the pages of this journal and by the academic and technical degrees held by its members and sought by student members. Our unique (and intended) structure, however, puts us at the center of applied environmental science. The Society's membership represents a broad spectrum of environmental stakeholders, including academic institutions of higher learning and research, government research laboratories, regulatory science and policy offices, private-sector testing laboratories, consulting firms, industries and nongovernmental organizations (NGOs). Through this collection of interrelated interests and missions, "SETAC's science" has immediate and direct application.

With noted exceptions (LCA advisory group, Congressional Science Fellow program, SETAC home page), most of SETAC's interactions and communications to date have been within the Society and between our members and their organizations. No doubt, *Environmental Toxicology and Chemistry* (*ET&C*), annual meetings, regional chapters, workshops, and *SETAC-News* have been, and will continue to be, mainstays of the Society. Demand and opportunity are growing, however, for SETAC to engage its science in the broader playing fields of environmental policy, decision-making, and general education. This aspect was also envisioned by the founders of SETAC: It is written in our charter and is inherent in our structure and underlying philosophy of representation from academia, government, and business in all our activities. Expanding our interactions has not been our first priority, but such a broader role seems inevitable for SETAC.

It is hard to imagine "the environment" losing its place as a high-priority public issue any time soon. The Society's science has an important role to play in many aspects of this issue, and we are experiencing increased pressure to engage it. This pressure comes from within SETAC; many of our individual members, their employers, and our sustaining members represent "user groups" for SETAC's science. Along with increasing visibility and credibility come increasing numbers of requests from environmental stakeholders outside the membership for our society to more fully engage its science. To be responsive will mean going beyond our traditional and very successful routes of communicating our science among SETAC members. It means broadening the availability of our science as well as extending its interpretation and communication to a wider audience. It means not only entering the debates among scientists but also educating the general public and the nonscientist policy-

and decision-makers. While we must be practical, realizing that science is only one component of any public environmental issue, we must also recognize that unless we bring our science to the discussion (that is, not just making it available but also delivering it effectively), it cannot become a key factor in decisions and future directions.

The Board of Directors, European Council, Executive and Assistant Executive Directors, committee chairs, editors, annual meeting organizing committees, and others in leadership roles in the Society are attuned to finding ways for SETAC to bring its science to policy-makers, decision-makers, educators, and the public as well as to scientists in related fields. Some of the expansions and new initiatives that are being discussed and/or are under development include the following:

- Producing technical issue papers, white papers on high-profile (hot) topics such as endocrine disrupters and multiple stressors;
- Expanding SETAC advisory groups to deliver sound multidisciplinary science and multistakeholder perspectives in additional hot topic areas;
- Increasing our active involvement in overarching areas, such as ecological risk assessment and risk management, that integrate science, policy, and decision-making;
- Expanding the SETAC home page and other on-line services;
- Identifying experts and expert groups within SETAC to serve as timely technical resources for communication with regulatory, legislative, and business leaders;
- Enhancing the Congressional Science Fellow program;
- Developing new SETAC journals and publications that target different audiences;
- Increasing interactions with other closely aligned professional societies through workshops and short courses;
- Expanding annual meetings and other principal communication tools to encourage mutually beneficial interactions with other environmental stakeholder groups (e.g., state and local government groups, human health risk assessors, environmental engineers, environmental lawyers, science writers, and reporters);
- Collaborating with NGOs committed to bringing sound science to environmental decisions; and
- Expanding *ET&C*'s accessibility in Latin America and Eastern Europe.

Looking forward, SETAC appears destined to be a player on a broader field dealing with environmental issues. The balanced perspective and multidisciplinary approach that we are committed to bringing to environmental science will continue to be SETAC's trademark. Our charter, underlying philosophy, and dedicated volunteer spirit will continue to serve us well as we move forward, advancing the fields of environmental toxicology, environmental chemistry, and hazard/risk assessment, following through on our own initiatives to engage our science

more broadly, and responding to what will surely be a growing and diverse array of user groups that will come to us for information, collaboration. and action. It is going to be an exciting time for SETAC!

As you read the articles in this issue of *ET&C*, as you prepare your own manuscript for *ET&C*, as you participate in the upcoming annual meeting, your regional chapter meeting, or a SETAC workshop, consider the growing and diverse "SETAC audience" and how your sound science (and SETAC's more broadly) might be engaged to its fullest. Then take the initiative: Bring your recommendation forward, be the SETAC champion for that idea, and help make it happen.

Daniel M. Woltering, Ph.D.
*President, Society of Environmental
Toxicology and Chemistry*
Principal, ENVIRON Corporation
Arlington, Virginia

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Tri-Services Workshop

INVESTIGATIONS OF ENZYMATIC ALTERATIONS OF 2,4-DICHLOROPHENOL USING 13C-NUCLEAR MAGNETIC RESONANCE IN COMBINATION WITH SITE-SPECIFIC 13C-LABELING: UNDERSTANDING THE ENVIRONMENTAL FATE OF THIS POLLUTANT

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Abstract—The biodegradation of 13C-labeled 2,4-dichlorophenol (DCP labeled at the C-2 and C-6 positions), in the presence and absence of natural organic matter (NOM), by the white-rot fungus *Phanerochaete chrysosporium*, was examined using 13C-nuclear magnetic resonance (NMR). Using this method permitted the chemistry occurring at or near the labeled site to be followed. The formation of alkyl ethers and alkene ethers was observed. No aromatic by-products were detected, indicating that aromatic compounds are quickly degraded. Examining the reaction with time shows the exponential removal of 2,4-DCP and the consequential formation of labeled by-products, whose concentration reaches a maximum just before all 2,4-DCP is consumed. After this, the by-products degrade exponentially. The presence of NOM causes 2,4-DCP to be removed from the aqueous phase more quickly than in its absence and also causes the by-products to reach their maximum concentration much earlier. Degradation of the by-products occurs at a much greater rate in the presence of NOM. One hypothesis for this behavior is that the NOM interacts with 2,4-DCP and its by-products, allowing them to be incorporated into the fungal biomass. 13C-nuclear magnetic resonance spectra of the fungal biomass after NaOH extraction show the presence of alkanes and a small amount of 2,4-DCP.

Keywords—2,4-Dichlorophenol   Biodegradaton   Enzymatic alteration   *Phanerochaete chrysosporium*   13C-nuclear magnetic resonance

INTRODUCTION

In order to effectively assess and understand bioremediation strategies for various pollutants, it is important to examine the biotransformation processes which occur and the environmental fate of pollutants [1] and their by-products. One concern regarding the by-products is that they may be more or just as toxic as the parent compound [1]. This is especially important if the by-products are more hydrophilic than the parent molecule and therefore have a greater mobility in soils, water, and sediments. On the other hand, the pollutant molecules or their by-products could be sequestered through covalent bonding via addition or oxidative coupling reactions with organic macromolecular material in soils and sediments. Covalent bonding could change the chemical and physical behavior of the pollutant, especially if this binding is irreversible, resulting in the immobilization of the pollutant and its by-products [2–4]. Noncovalent associations between the parent molecule or the by-products and the organic macromolecule are also possible and have potential for influencing the chemical and physical behavior of the pollutant [5]. Such associations include ion exchange, hydrogen bonding, protonation, charge transfer, ligand exchange, coordination through metal ions, van der Waals forces, and hydrophobic bonding. Therefore, to truly understand the biodegradation of pollutants, it is necessary to have knowledge regarding their environmental fate, the mechanistic pathways,

the formation of by-products, and the possible covalent and noncovalent interactions of the pollutant molecules with naturally present organic matter. Clearly, bioremediation assessment requires much more than merely monitoring the disappearance and mineralization rate of the pollutant compounds, which are typically the only measurements acquired [6–10].

Traditionally, biodegradation by-products have been examined by solvent extraction methods followed with gas chromatography (GC) techniques and gas chromatography–mass spectrometry (GC–MS). Unfortunately, many degradation by-products and mechanistic pathways are unknown because the by-products are too polar or are not volatile enough for analysis by GC methods. However, a new method has been devised by Hatcher et al. [5] which overcomes these problems and is an extension of previously employed methods for examining complex natural processes [11–15]. This method involves labeling molecules with nuclear magnetic resonance (NMR)-sensitive nuclei in combination with standard NMR techniques. The NMR-sensitive nuclei labels serve as a flag to monitor chemical changes at or near the labeled sites in the reacting molecule. For example, in the case of 13C nuclei, the natural abundance of 13C is only 1.1%, so the signals from the 13C-labeled sites will be approx. 100 times more intense than the signals from the naturally present 13C nuclei. The signals from the naturally present 13C nuclei will be of such low intensity that they will be lost in the baseline noise. Thus, the chemistry occurring at or near the labeled sites can be exclusively monitored by 13C-NMR.

Hatcher et al. [5] and Bortiatynski et al. [16] have applied this technique to successfully characterize the coupling of 2,4-

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dichlorophenol (2,4-DCP), ¹³C-labeled in the C-2 and C-6 positions or in the C-1 position, to a Minnesota peat humic acid in the presence of horseradish peroxidase, an oxidoreductive enzyme. They obtained ¹³C-NMR spectra consisting of signals which were interpreted as being representative of (1) 2,4-DCP polymerizing with itself, (2) 2,4-DCP bound to humic acid through ester linkages, (3) 2,4-DCP bound to humic acid through phenolic ether linkages, and (4) 2,4-DCP bound to humic acid through carbon–carbon linkages. They found that the 2,4-DCP reacts with the humic acid only in the presence of enzyme and that much of the chemistry occurring can be explained by reaction at the chlorinated sites of 2,4-DCP. This indicated that removal of the chlorine atom occurred before or was coincident with the binding to humic acid. Bortiatynski et al. [16], using ¹³C-labeled phenol in the C-1 position, have further demonstrated that covalent coupling occurs only with humic acid in the presence of enzymes. The NMR signals present after the enzymatic reaction indicate the formation of ether, ester, and C–C linkages. Bortiatynski et al. [16] were also able for the first time to directly assess noncovalent bonding associations between the phenol and the humic acid using this ¹³C-labeling approach. This was accomplished by measuring spin-lattice relaxation times (T_1) of the ¹³C-labeled phenol. Using T_1 data, they were able to demonstrate that a linear relationship existed between the amount of associated ¹³C-phenol and humic acid. From this data they were able to calculate the concentration of ¹³C-phenol that was noncovalently associated with the humic acid. The T_1 data were also used to determine a fractal dimension for the humic acid surface, which suggested that the reaction surface of the humic acid is intermediate between a plane and a line. Thus, ¹³C-NMR in combination with ¹³C-labeling has been shown to be useful in examining the covalent and noncovalent interactions of specific molecules and natural organic macromolecular material.

Using this approach, we took this technique one step further and examined the degradation of 2,4-DCP by the white-rot fungi *Phanerochaete chrysosporium*. This species has been shown to mineralize polychlorinated phenols present in a pure culture [10,17], in contaminated wood samples [18], and in contaminated soil samples [7,8,19,20]. *Phanerochaete chrysosporium* has also been shown to degrade and mineralize a wide array of other aromatic, recalcitrant pollutant compounds [21–23], such as polycyclic aromatic hydrocarbons; chlorinated aromatic hydrocarbons; pesticides such as DDT, chlordane, and lindane; and munitions such as 2,4,6-trinitrotoluene. *Phanerochaete chrysosporium* degrades these compounds with the nonselective enzymes lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP). Excretion of these enzymes is stimulated by deprivation of carbon, nitrogen, and sulfur sources. When degrading lignin-containing plant material, these fungi use LiP and MnP to chemically breakdown the lignin in order to expose cellulose, which they use as a carbon source. It has been well demonstrated that the isolated LiP and MnP enzymes are capable of degrading and mineralizing polychlorinated phenols [9,17,24,25].

Phanerochaete chrysosporium holds several advantages as a potential species for bioremediation as compared to other typical microbes used for such purposes. Because LiP and MnP are both nonspecific exoenzymes, white-rot fungi are capable of degrading not only a wide variety of compounds but also complex mixtures of pollutants [23]. This is unlike many microbes commonly used for bioremediation, which are specifically adapted to degrade only one compound or are able to contribute

to only one step of the degradation process. Because of this, a population of numerous microbial species is often required to mineralize a pollutant. Also, because *P. chrysosporium* utilizes exoenzymes for degradation, it is capable of tolerating much higher concentrations of toxic material than most microbes [23]. Finally, because LiP and MnP production and excretion are stimulated by nutrient deprivation rather than the concentration of the pollutant, the kinetics of pollutant degradation is not dependent on the concentration of the pollutant. Thus, because of its versatility, *P. chrysosporium* seems to be an ideal organism for use in bioremediation strategies of recalcitrant pollutants present in contaminated soils [23].

The purpose of this study was to extend the previous research of Hatcher et al. [5] and Bortiatynski et al. [16,26], which examined the use of oxidoreductive enzymes for the degradation of 2,4-DCP. The only difference was that in this study, the degradation reactions, both in the absence and presence of natural organic matter (NOM), were initiated by a living culture of *P. chrysosporium*. This was done in order to gain a better understanding of the reaction mechanisms involved and the by-products formed during the biodegradation of ¹³C-labeled 2,4-DCP (labeled in the C-2 and the C-6 positions) by this species of white-rot fungi.

EXPERIMENTAL

Materials

The labeled ¹³C-2,4-DCP was prepared from phenol labeled with 100% ¹³C at the C-2 and C-6 positions (Cambridge Isotope Laboratories, Woburn, MA, USA). A modified version of the chlorination procedure by Ebel et al. [27] was utilized in which the chlorine gas was delivered to the reaction vessel directly rather than by the suggested method of chlorine generation from MnO_2 and HCl. This modification allowed for a more controlled rate of addition of the chlorine gas. The reaction produces a mixture of mono-, di-, and trichlorinated phenols, but by careful selection of reaction conditions, the product yield of 2,4-DCP could be maximized to 60%. Complete separation of all the various mono-, di-, and trichlorinated phenol isomers was attained by high-performance liquid chromatography (HPLC) on a 4.6- × 150-mm liquid chromatograph (LC-18, Supelco, Bellefonte, PA, USA). The mobile phase was 50:50 water:methanol adjusted to pH 4 with formic acid.

The NOM was obtained from E.M. Perdue (Georgia Institute of Technology, Atlanta, GA, USA) and was isolated from the Suwannee River. This NOM was used because of its solubility in water.

Fungal culture and addition of 2,4-DCP

Cultures of *P. chrysosporium* (strain BKM-F-1767; ATCC 24725) were maintained on supplemented malt agar slants; the medium used is described elsewhere [28]. Spore production in the slants required 2 to 5 d of growth at 39°C. Spores (conidia) were prepared by suspension in sterile water followed by passage through sterile glass wool to free them of contaminating mycelia. Shallow stationary cultures (10 ml) were grown in rubber-stoppered, 125-ml Erlenmeyer flasks at 39°C under 100% oxygen. They were flushed with oxygen at the time of inoculation and again on day 3. Composition of the aqueous, nitrogen-limited media for the stationary cultures is described in detail elsewhere [28]. Mycelial growth under the nitrogen-limited conditions was stopped by day 2, and ligninase activity appeared in the extracellular fluid on day 4.

2,4-dichlorophenol
White-rot fungi (6 hrs)
No NOM present
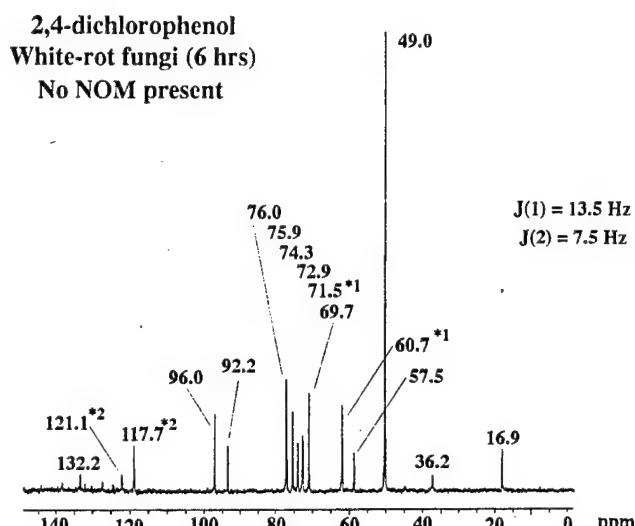
Fig. 1. ¹³C-nuclear magnetic resonance (NMR) spectrum of the fungal aqueous fraction 6 h after addition of ¹³C-labeled 2,4-dichlorophenol. No natural organic matter (NOM) is present in sample.

On day 6 the cultures were flushed once again with oxygen. To 28 culture flasks, 0.19 ml of 2.129 g/L stock solution of ¹³C-labeled 2,4-DCP was added so that each culture contained a 2,4-DCP concentration of 40 mg/L. The 2.129 g/L stock solution of 2,4-DCP contained approx. 5% methanol to ensure solubility of 2,4-DCP at the solution pH of approx. 7. To half of these cultures 0.22 ml of Suwannee River NOM stock solution was added to provide a final NOM concentration of 50 mg/L. Control solutions were also made which contained only fungal culture, fungal culture and NOM, NOM alone, and 2,4-DCP alone. Two replicate cultures containing only 2,4-DCP and 2,4-DCP with NOM were sampled at 30 s, 6 h, 12 h, 30 h, 48 h, 168 h (1 week), and 336 h (2 weeks). Cultures were flushed every 3 d with 100% oxygen. Upon sampling, sodium azide stock solution was added to form a 0.1-M solution to stop fungal growth and enzyme activity. The two replicate cultures were added together and then homogenized with a tissue grinder. Biomass material was separated with a table-top centrifuge, and the supernatant was removed with a pipette and filtered with filter paper before ¹³C-NMR analysis. All biomass and supernatant samples were frozen until NMR analysis. Control replicates were processed in an identical manner.

Biomass samples were processed by dissolving the biomass pellet in 0.5 M NaOH directly in a 10-mm NMR tube (pH = 12). The NMR tube was spun in a table-top centrifuge for 30 min. before NMR analysis. After NMR analysis, the sample was acidified with 0.5 M HCl (pH = 2) and respun on the table-top centrifuge before a second analysis by NMR.

Nuclear magnetic resonance spectroscopy

The NMR spectra were obtained on a Bruker AMX 360 NMR spectrometer with a ¹³C resonance frequency of 90.55 MHz. Two types of NMR spectra were obtained in this study, proton decoupled ¹³C and distortionless echo polarization transfer (DEPT).

The ¹³C NMR spectra were obtained at 303°C using a standard inverse-gated pulse sequence. The experimental parameters were (1) sweep width of 22,727 Hz, (2) a 30° pulse width, and (3) a recycle delay of 20 s. The data were processed with 5-Hz line broadening.

A standard DEPT sequence was used to determine degree of protonation. For all DEPT experiments, a coupling constant of 145 Hz was used to calculate the delay time for signal evolution, and a delay of 20 s between pulses was used to ensure complete relaxation.

RESULTS AND DISCUSSION

Aqueous fraction samples

Analysis of the supernatant from degradation experiments by ¹³C-NMR demonstrates that 2,4-DCP is quickly degraded by the fungi into distinct hydrophilic compounds. This is indicated by the reduction of the NMR signals for 2,4-DCP and the emergence of several new signals after 6 h of reaction (Fig. 1) as compared to the ¹³C-NMR spectrum of 2,4-DCP before its addition to the fungal culture (Fig. 2). The ¹³C-NMR spectrum of 2,4-DCP contains two doublets at 117.7 and 121.1 ppm, each with a ¹³C–¹³C two-bond coupling constant of 7.5 Hz. The singlet at 49.0 ppm is from methanol, which was added to enhance the solubility of 2,4-DCP.

Table 1 presents the chemical shift positions, number of attached protons (as determined from DEPT experiments), coupling values, and tentative assignments for all signals. Identi-
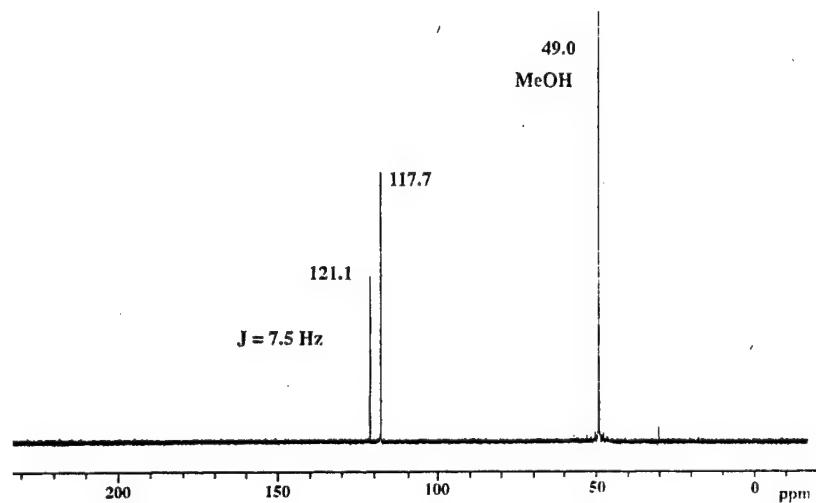
Fig. 2. ¹³C-nuclear magnetic resonance (NMR) spectrum of 2,4-dichlorophenol before inoculation with white-rot fungi. Methanol was present to increase 2,4-dichlorophenol solubility.

Table 1. Chemical shift data, distortionless echo polarization transfer (DEPT) data, coupling constant data, and tentative identification for ^{13}C-NMR signals in Figure 1

Chemical shift (ppm)[a]	Degree of protonation[b]	J_{C-C} (Hz)	Comments
16.9	CH₃		Alkane
36.2			Alkane (background signal)
49.0	CH₃		Methanol
57.5	CH₂		Ethanol
60.7	CH₂	13.5	Alkyl ether
69.7	CH		Alkyl ether
71.5	CH	13.5	Alkyl ether
72.9	CH		Alkyl ether
74.3	CH		Alkyl ether
75.9	CH		Alkyl ether
76.0	CH		Alkyl ether
92.2	CH		Alkene ether
96.0	CH		1-Methoxypropene(?)
117.7		7.5	2,4-Dichlorophenol
121.1		7.5	2,4-Dichlorophenol
132.2			Background signal

[a] Relative to TMS.
[b] DEPT data: 45° flip angle for all carbons with attached protons, 90° flip angle for methine carbons, 135° flip angle to distinguish between methyl and methylene carbons.

fiable compounds are methanol (present in unlabeled form from the 2,4-DCP stock solution) at 49.0 ppm, ethanol at 57.5 ppm, and 2,4-DCP with two doublets at 117.7 and 121.1 ppm (Fig. 1). Except for the signals due to methanol, ethanol, 2,4-DCP, and an unidentified alkane at 16.9 ppm, all other signals appear within the region of 55 to 100 ppm. The absence of any major signals, other than those of 2,4-DCP, in the region of 100 to 165 ppm demonstrates that there are no, or very low, concentrations of aromatic or alkene structures carrying the ^{13}C label in the aqueous fraction. This indicates that the aromatic ring structure of 2,4-DCP is quickly broken apart as the chemical shift of the labeled carbons shift to nonaromatic regions of the spectrum.

Signals in the region of 60 to 80 ppm arise from alkyl ethers [29]. As indicated by DEPT experiments and illustrated in Figure 3, the signals in the region of 69 to 80 ppm are from [13]C-
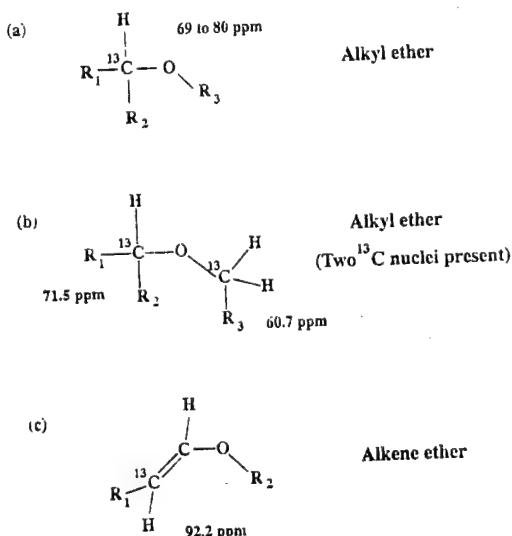
Fig. 3. Chemical structures of (a) alkane ether, (b) alkane ether containing two ^{13}C nuclei, and (c) an ether-substituted alkene.
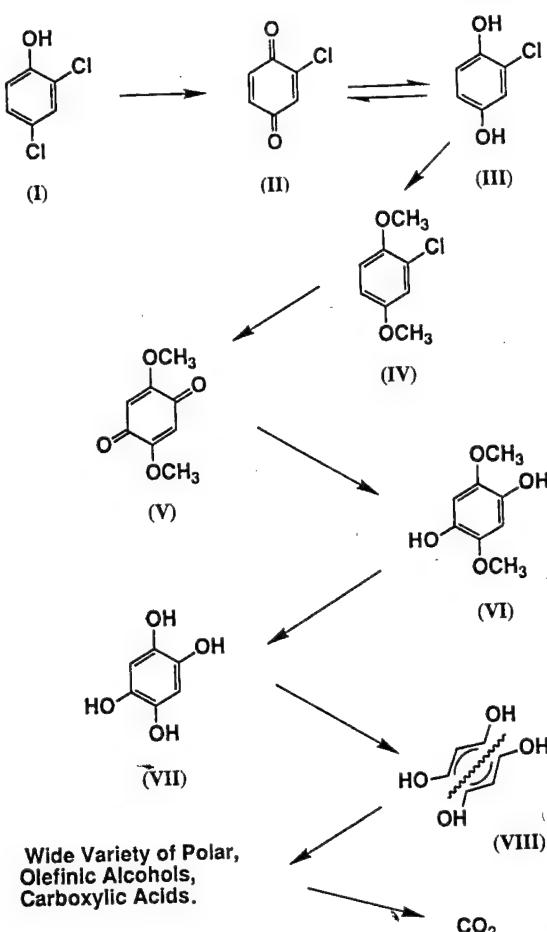
Fig. 4. Pathway for the oxidation and eventual aromatic ring cleavage of 2,4-dichlorophenol by white-rot fungi. Adapted from Joshi and Gold [17], Hammel and Tardone [24], Valli and Gold [25], Cain [30], and Odier and Artaud [31].

labeled carbons containing only one proton, while the signal at 60 ppm is from a ^{13}C-labeled carbon containing two protons. The presence of alkyl ethers containing ^{13}C-labeled carbons demonstrates that chlorine is removed from the C-2 site of DCP, followed by methoxylation or hydroxylation of this site, and then undergoes a coupling reaction with another carbon atom. The two signals at 60.7 and 71.5 ppm arise from the formation of an alkyl ether in which both carbon atoms bonded to the oxygen are ^{13}C-labeled (Fig. 3b). This is confirmed by the carbon–carbon coupling of 13.5 Hz present in each signal. Two-bond carbon–carbon coupling constants are usually within the range of 7.6 to 15.2 Hz [29]. The signals at 92.2 and 96.0 ppm are presumably due to ether-substituted alkenes of the type shown in Figure 3c. For example, the β-carbon of (E) 1-methoxypropene has a chemical shift value of 96.0 ppm [29].

The formation of alkyl and alkene ethers during the degradation of halogenated aromatic compounds by *P. chrysosporium* has not been previously documented. The pathway for the dehalogenation of polychlorophenols by isolated LiP and MnP has been examined by Hammel and Tardone [24], Valli and Gold [25], and Joshi and Gold [17]. In these studies, the dehalogenation and degradation by-products were analyzed with GC–MS methods and, in some instances, HPLC techniques. The general degradation pathway is believed to commence with initial oxidation of the 2,4-dichlorophenol (I) by LiP or MnP, resulting in the dehalogenation and oxidation in the C-4 position, forming
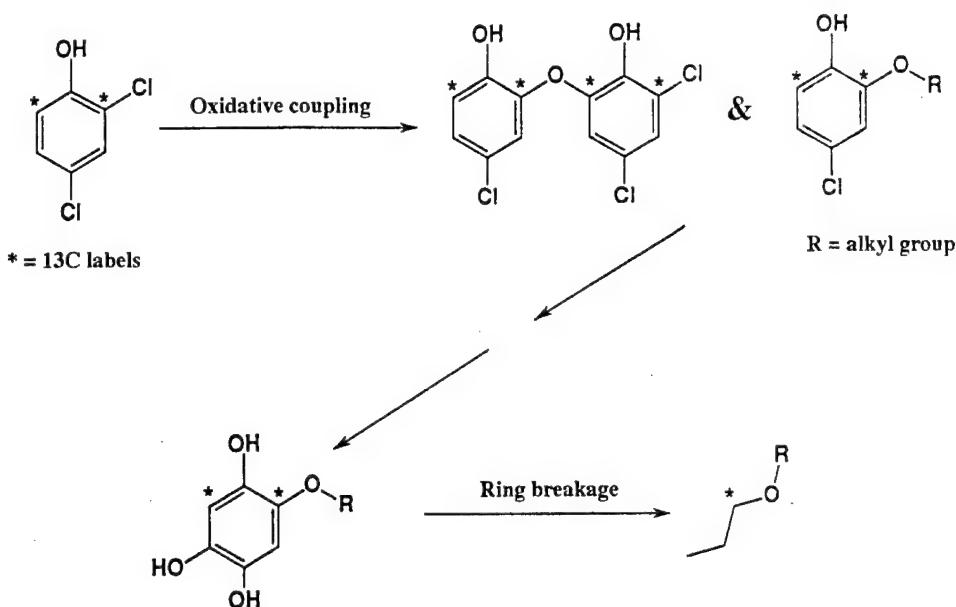
Fig. 5. Proposed pathway leading to the formation of alkyl and alkene ethers from 2,4-dichlorophenol through oxidative coupling.

2-chloro-1,4-benzoquinone (II), as shown in Figure 4. 2-chloro-1,4-Benzoquinone (II) is reduced to 2-chloro-1,4-hydroquinone (III), which then is methylated to 2-chloro-1,4-dimethoxybenzene (IV). A second dehalogenation and oxidation occurs, removing the chlorine atom in the C-2 position, forming 2,5-dimethoxy-1,4-benzoquinone (V). This compound then is reduced to 2,5-dimethoxy-1,4-hydroquinone (VI), which is converted to 1,2,4,5-tetrahydroxybenzene (VIII). At this point, the polyhydroxylated aromatic ring ruptures between two adjacent hydroxylated carbons, forming a muconate compound (VIII) [30,31]. Additional bonds in the muconate compound can continue to be broken until CO_2 is formed as the final product. Our results show that coupling reactions can also occur once the chlorinated carbon is dehalogenated and oxidized to form hydrophilic alkyl ethers by a possible pathway shown in Figure 5. Since our ^{13}C-NMR results show only the presence of alkyl ethers, alkene ethers, and 2,4-DCP, they indicate that dehalogenation of the polychlorophenol compounds, once it begins, is a fairly complete reaction, i.e., the quinone or polyhydroxylated benzene is not present at any appreciable concentration because it quickly continues to be oxidized and dehalogenated until it forms a much less reactive species or is mineralized.

No difference was found between the ^{13}C-NMR spectrum in which no NOM was present (Fig. 1) and the spectrum in which Suwannee River NOM was present (not shown) during the fungal degradation process. The ^{13}C-NMR signals present are all sharp and distinct, indicating that the ^{13}C nuclei are in discreet chemical environments. This could indicate that specific products are formed rather than numerous, random polymeric-type compounds. This is consistent with studies of Hammel and Tardone [24] and Valli and Gold [25], who detected only the species resulting from the phenolic monomer rather than dimer or polymeric compounds during *P. chrysosporium* biodegradation of 2,4,6-trichlorophenol and 2,4-DCP, respectively. Joshi and Gold [17] also detected primarily monomeric by-products from the fungal degradation of 2,4,5-trichlorophenol, although they did observe trace amounts of 2,2'-dihydroxy-3,3',5,5',6,6'-hexachlorobiphenyl. This demonstrates that 2,4-DCP or its by-products are not forming a wide variety of different types of covalent bonds with 2,4-DCP or dissolved organic matter but rather with

discreet, specific compounds. This is in contrast to the research of Hatcher et al. [5] and Bortiatynski et al. [16], in which ^{13}C-labeled 2,4-DCP formed numerous bonds with a Minnesota peat humic acid or through self-polymerization in the presence of horseradish peroxidase, giving rise to large, broad ^{13}C-NMR signals in the aromatic region.

All the signals in the NMR spectrum of the aqueous fraction are due to the ^{13}C labels, except for the methanol signal at 49.0 ppm, which was introduced into the sample from the addition of the 2,4-DCP stock solution, and the two small signals detected in the control samples at 37.0 and 131.9 ppm. Except for the small signals at 37.0 and 131.9 ppm, the NMR spectra of the control samples, with and without NOM present, are devoid of signals.

Loss of ^{13}C label as a function of time

Figures 6 through 9 show the quantitative disappearance of 2,4-DCP and the formation and eventual disappearance of reaction products as a function of time for aqueous samples in
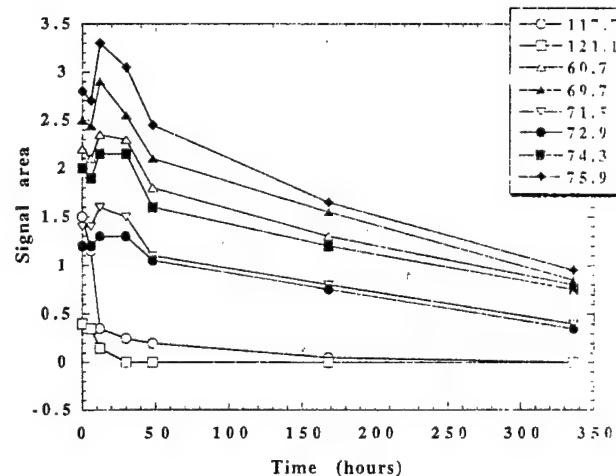
Fig. 6. Signal intensities of ^{13}C-labeled 2,4-dichlorophenol and alkane ethers, in the absence of natural organic matter (NOM), as a function of time.
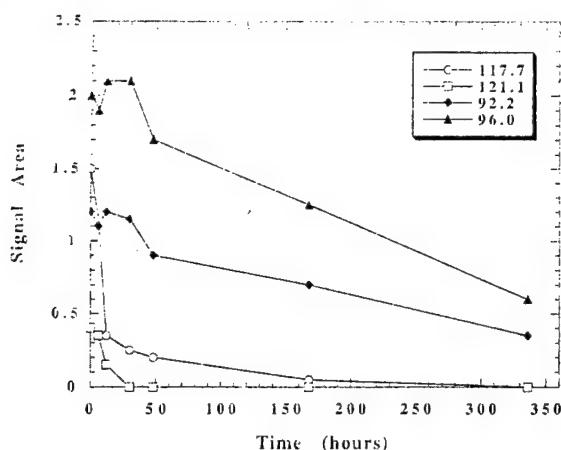
Fig. 7. Signal intensities of 2,4-dichlorophenol and ether-substituted alkenes, in the absence of natural organic matter (NOM), as a function of time.

Fig. 8. Signal intensities of ^{13}C-labeled 2,4-dichlorophenol and alkane ethers, in the presence of natural organic matter (NOM), as a function of time.

the absence and presence of NOM. respectively. All signal areas were measured relative to the area of the methanol signal. The area of the methanol signal in each spectrum remained constant throughout the entire experimental period; therefore, it was used as an internal reference. The signal areas are presented in Figures 6 through 9. As seen in Figure 6, 2,4-DCP decays exponentially. The reason the areas of the 2,4-DCP signals are not equivalent in these figures is because the use of a 20-s pulse delay did not provide sufficient time for the C-2 ^{13}C-labeled carbon to relax. Increasing the pulse delay to 50 s provided enough time for relaxation, and both signal areas were then equivalent.

As it degrades, the concentration of the alkyl ethers (Fig. 6) and the alkene ethers (Fig. 7) increase, reaching a maximum at 12 h. After approx. 30 h, the concentration of these products begins to decrease. Comparison of the total signal area (except that of methanol, which was used as the reference) at 0 h and at 336 h (14 d) shows that 58% of the ^{13}C label in the samples not containing NOM has been removed from the aqueous phase, presumably due to mineralization or binding to the solid residues. This is fairly consistent with the conclusions of Valli and Gold [25], who showed that 50% of 2,4-DCP was mineralized in 30 d, and Joshi and Gold [17], who showed that within 15 d, approx. 57% of 2,4,5-trichlorophenol had mineralized. In both cases, nitrogen-limited stationary phase cultures were used. Lin et al. [9] have shown for a pure culture of *P. chrysosporium* that the degradation rate for pentachlorophenol is dependent on the extracellular enzyme concentration and the cell mass concentration and that depending on these variables, 33 to 70% of pentachlorophenol is mineralized in 22 d.

[13]C spectra of the fungal biomass after NaOH extraction did not detect the presence of any labeled material, supporting the idea that loss of ^{13}C nuclei in a pure aqueous phase is due primarily to mineralization and that binding to the fungal biomass, which was almost completely solubilized by NaOH, is minor or negligible.

Examination of Figures 8 and 9 demonstrates that the presence of NOM has a pronounced effect on the degradation rate of 2,4-DCP and the formation and degradation rate of the by-products. Although it is difficult to ascertain quantitatively how the degradation of 2,4-DCP differs in the presence of NOM, it is possible to state that the 2,4-DCP is no longer detected at 48 h, while in the absence of NOM, 2,4-DCP is detected up to 168
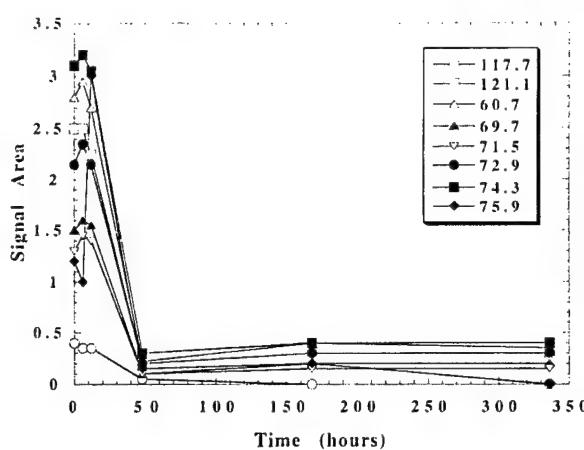
h (1 week). Another observation is that the maximum concentration of by-products occurs at 6 h in the presence of NOM, as opposed to 12 h when NOM is absent. In the presence of NOM, the by-products dramatically decrease within 48 h to very low concentrations (Fig. 6–9). A possible explanation for the difference in reaction rates when in the presence of NOM is that the NOM sorbs or reacts with the 2,4-DCP and by-products such that a wide variety of compounds are formed, resulting in extensive dispersion of chemical shifts and reduction of intensity of the corresponding signals. An additional hypothesis is that the NOM is involved with incorporating the ^{13}C-labeled compounds into the fungal biomass. Figure 10 presents the 30-s ^{13}C-NMR spectra of the NaOH extract of the fungal biomass sample and the spectrum of this extract after acidification with HCl. The NaOH extraction caused precipitated humic and fulvic acids to dissolve. Acidification of this extract then would precipitate humic acids. In this manner, any ^{13}C label associated with humic acids will be detected only in the NaOH extract, but not in the acidified extract. In the NaOH extract, three prominent signals in the alkane region are detected at 29.8, 30.0, and 30.3 ppm. Also detected is a small signal at 123.2 ppm, presumably from 2,4-DCP, which has signals at this chemical shift at pH values above 7 [16]. When the NaOH extract is acidified, the alkane signals remain unchanged, but a new, small signal at 135.02 ppm appears while the signal at 123.2 ppm is absent.
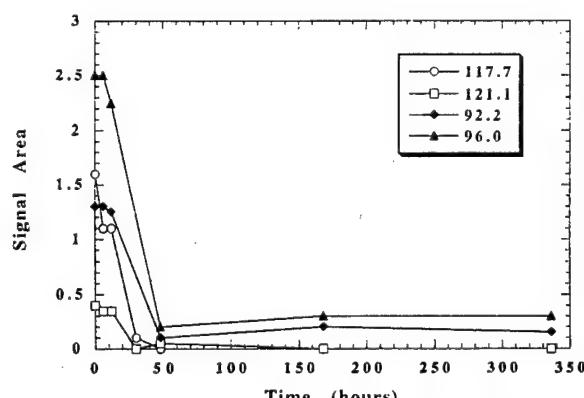
Fig. 9. Signal intensities of 2,4-dichlorophenol and ether-substituted alkenes, in the presence of natural organic matter (NOM), as a function of time.
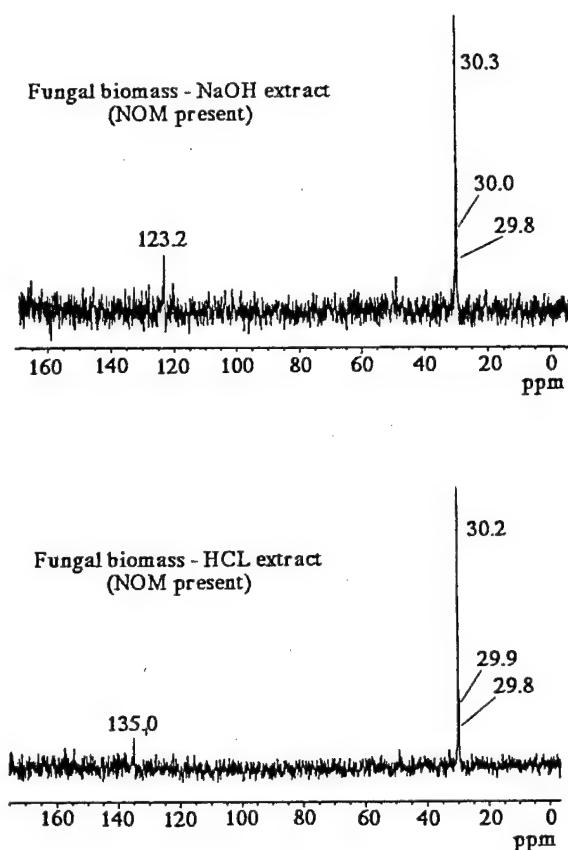
Fig. 10. ^{13}C-nuclear magnetic resonance (NMR) spectra of the fungal biomass fraction after NaOH extraction and then after HCl extraction following the previous NaOH extraction. Biomass sample was in the presence of natural organic matter (NOM).

After 30 h, none of these signals are present. Signals were not detected in any of the biomass fractions of the controls which lacked the ^{13}C-labeled 2,4-DCP.

CONCLUSIONS

Through the use of ^{13}C-labeled 2,4-DCP and ^{13}C-NMR spectroscopy, the biodegradation of 2,4-DCP by *P. chrysosporium* has been examined, both in the absence and presence of NOM from the Suwannee River. The absence of signals in the aromatic region of the ^{13}C-NMR spectrum shortly after addition of 2,4-DCP illustrates that the aromatic ring structure of 2,4-DCP is quickly destroyed and new products are formed. Alkyl ethers were found to be major products, in addition to alkene ethers. These reaction products, which have not been detected before for this reaction, indicate that the chlorine atom is removed at the ^{13}C-labeled site, presumably followed with a hydroxylation or methoxylation, and then followed with a coupling reaction to form an ether. We have shown that these products are formed during the degradation of 2,4-DCP by *P. chrysosporium*. We have shown that as the concentration of 2,4-DCP decreases, the concentration of the new products increases until all the 2,4-DCP is depleted. At this point, the concentration of the products begins to decrease with time. The presence of NOM was found to strongly affect the reaction rates, i.e., the disappearance of 2,4-DCP and the formation and removal of products, by apparently removing them from the aqueous fraction and incorporating them into the fungal biomass.

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MICROBIAL MINERALIZATION OF ORGANIC COMPOUNDS IN AN ACIDIC AGRICULTURAL SOIL: EFFECTS OF PREADSORPTION TO VARIOUS SOIL CONSTITUENTS

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Abstract—This study investigated the interactions between organic chemicals and components of the soil matrix and their effects on subsequent microbial mineralization kinetics. Five ^{14}C-labeled chemicals (anionic, cationic, and nonionic surfactants) were aseptically sorbed to montmorillonite, kaolinite, illite, sand, humic acids, and fulvic acids. Small amounts of these sorbed chemicals were dosed to an acidic, sludge-amended agricultural soil (Rossmoyne) to a final added chemical concentration of 50 ng g^{-1}. Controls received the same final added concentration of the chemicals in water. The ratio of sorbed chemical to soil was kept low to minimize changes to the soil mineralogy, chemistry, and microbiology. Microbial mineralization of the chemicals to ^{14}CO$_2$ was measured over a period of 60 to 70 d, and the data were fitted to first-order and 3/2-order mineralization models. Association with the soil constituents inhibited the mineralization of the chemicals in the following rank (from least to greatest effect): controls ≈ sand < kaolinite < illite < montmorillonite ≈ humic acids < fulvic acids. These experiments demonstrated that interactions with some soil constituents (kaolinite, illite, and sand) had little effect on the microbial metabolism of these chemicals, while montmorillonite, humic acids, and especially fulvic acids significantly decreased the bioavailability of the chemicals to the microbial community. The first group of soil constituents had little influence on the mineralization kinetic parameters, whereas the latter significantly reduced at least one of the parameter estimates. The parent soil, possibly via interactions with its mineral surfaces, also had effects on the degradation of the chemicals, since soil microbial biomass and physiological activity were not correlated with any of the mineralization kinetic parameter estimates. These experiments demonstrate that the environmental form of a chemical has a significant influence on its eventual microbial metabolism and is an important parameter to consider when investigating the fate of chemicals in soil environments.

Keywords—Biodegradation   Soils   Bioavailability   Desorption

INTRODUCTION

The biodegradation of a xenobiotic chemical in terrestrial surface environments is governed by several physical/chemical factors that influence the availability of that chemical to the microbial populations responsible for its catabolism. Most chemicals will interact in some manner with part of the soil matrix, owing to the rich diversity of chemical surfaces and moieties present in or on soil minerals [1,2], their iron oxide and organic coatings [3], and in soil colloids [4]. Some of these interactions may enhance the biodegradation of the chemical, while others may retard its degradation [5,6].

To understand the relative importance of these influences and determine mechanisms that control the biodegradation of chemicals in soils, it is valuable to understand the behavior of chemicals in environmental forms that mimic the forms that would exist in the soil environment. These forms would be predominately determined by the component(s) of the soil matrix with which a chemical interacts upon entry into the soil. To better understand the effects of these interactions, we examined the microbial mineralization of five ^{14}C-labeled organic chemicals (differing in charge, size, and functional group composition) that had been sorbed to six purified soil constituents. We added these chemical–soil constituent materials to a weathered agricultural soil and measured the microbial mineralization of the ^{14}C-labeled chemicals over time. When these treatments were compared to controls (in which the chemicals had been added to the soil in water), the differences in mineralization patterns could be attributed to interactions with the soil constituents. We could then compare the differences between the chemicals and the different environmental forms to determine what factors (chemical or soil) are important in determining the fate of molecules in soil environments. Furthermore, since we previously examined the fate of some of these chemical–soil constituent complexes in a natural woodlot soil [7], we evaluated the effects of soil type on the fate of these complexed chemicals. This additional set of observations allowed us to compare and interpret the data further, since the soils used were chemically and physically different. Therefore, this study is an expansion of the earlier work [7] and provides a broader perspective of the behavior of organic chemicals in different soil types.

The chemicals chosen for this study were amphipathic molecules (surfactants) that differ in size, functional group composition, and charge. We chose these chemicals to serve as models to examine the influences that these different chemical characteristics may have on bioavailability and microbial mineralization. Dodecyl linear alkylbenzene sulfonate (LAS) and sodium stearate were the anionic surfactant and soap studied; dodecyl linear alcohol ethoxylate (LAE) was the nonionic sur-

factant used; and stearyl trimethyl ammonium chloride (STAC) and dodecyl trimethyl ammonium chloride (C_{12}TMAC) were the cationic surfactants studied. The chemicals were aseptically sorbed onto purified montmorillonite, kaolinite, illite, sand, humic acids, and fulvic acids. The ^{14}C-labeled chemical–soil constituent complexes were added to an acidic, weathered, sewage-sludge-amended agricultural soil (Rossmoyne) at a low level (0.1 g was added to 8.0 g soil). This was done to minimize any disruption of native mineralogy and microbiology of the soil. The microbial production of $^{14}CO_2$ was measured over time, and the data were fitted to kinetic models. The kinetic estimates were used to evaluate differences between the chemicals and the soil constituents and were also compared to their desorption coefficients (K_d) and to the soil microbial activity and biomass.

MATERIALS AND METHODS

Soil and soil constituents

The sewage-sludge-amended agricultural soil was collected at a site owned by the Eastgate Sod Company, Loveland, Ohio, USA. It has been classified as a Rossmoyne soil [8]. The site had received approx. 4.6 Mg sewage sludge ha^{-1} yr^{-1} for the previous 5 years. During sampling, the sod was removed and the soil was taken at a depth of approx. 10 to 15 cm. The soil was kept on ice and returned to the laboratory. The soil was partially air-dried under a stream of sterile air and sieved (<2 mm). Clay mineralogy of the soil was determined on the <2-µm fraction using the method of Jackson (Jackson, M.L. 1975. *Soil Chemical Analysis: Advanced Course*, 2nd ed. Published by author, Madison, WI, USA.); total surface area was determined by the ethylene glycol monoethyl ether method of Heilman et al. [9]. Cation exchange capacity was measured using the method of Bascomb [10].

Several clay, mineral, and organic soil constituents were used in these experiments. Montmorillonite SWy-1, kaolinite, and illite were purchased from the Clay Mineral Society, University of Missouri, Columbia, Missouri, USA. Sea sand was collected near Daytona Beach, Florida, USA, and combusted as previously described [7]. Humic acids (HAs) were purchased from Aldrich Chemical Company, Milwaukee, Wisconsin, USA. Fulvic acids (FAs) were extracted from an alpine tundra soil [11,12] using standard methods [13] and purified twice over Dowex-50 exchange resin (H$^+$ form). The eluate was lyophilized and stored at −20°C.

Chemicals

[U-^{14}C-ring]Dodecyl linear alkylbenzene sulfonate (LAS) (specific activity of 67.8 µCi mg^{-1}) was purchased from New England Nuclear, Wilmington, Delaware, USA. [U-^{14}C-Ethoxy]dodecyl linear alcohol ethoxylate (LAE) (average of 8.5 ethoxy residues per molecule; specific activity of 5.4 µCi mg^{-1}) and [U-^{14}C]sodium stearate (specific activity of 196.8 µCi mg^{-1}) were purchased from Amersham, Arlington Heights, Illinois, USA. 1-[^{14}C]Stearyl trimethyl ammonium chloride (STAC) (specific activity of 12.5 µCi mg^{-1}) and 1-[^{14}C]dodecyl trimethyl ammonium chloride (C_{12}TMAC) (specific activity of 95 µCi mg^{-1}) were synthesized at the Procter and Gamble Company, Cincinnati, Ohio, USA. [U-^{14}C]Acetate (specific activity of 57 mCi mmol^{-1}) was purchased from Amersham. The radiochemical purity of the surfactants was greater than 98% (determined by thin-layer chromatography), except for sodium stearate, which had a purity
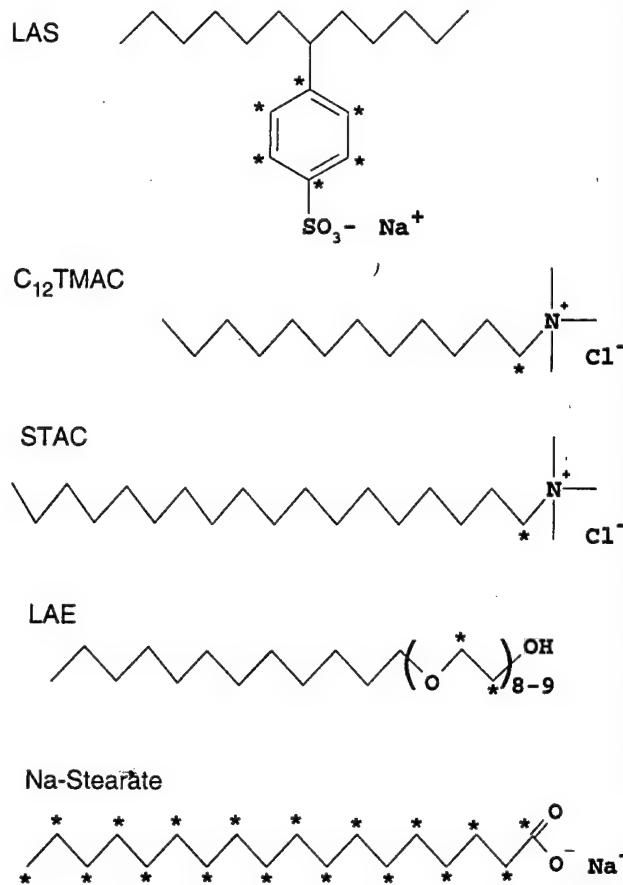
Fig. 1. Structural formulas for the chemicals used in this study. The location of the ^{14}C is indicated by an asterisk (*).

greater than 96%. All other chemicals used were reagent grade. Structural formulas of the surfactants are shown in Figure 1.

Sorption of chemicals to soil constituents

Dodecyl linear alcohol ethoxylate, LAS, STAC, C_{12}TMAC, and sodium stearate were aseptically bound to the montmorillonite, kaolinite, illite, sand, HAs, and FAs as described previously [7]. Briefly, the ^{14}C-labeled chemicals (0.145 mg) were combined with 4.0 g of the soil constituents in an excess of ethanol or methanol. These were mixed and subjected to gentle rotary evaporation at 40°C to remove the solvent. The sorbed chemical–soil constituent materials were further dried and stored over drierite. Following drying, the chemical–soil constituent complexes were aseptically ground to fine powders. The ratios of the ^{14}C-labeled chemical to the soil constituent were designed to provide a final chemical concentration of 50 ng g^{-1} soil when 0.1 g of the complex was dosed to 8.0 g of the soil.

The use of the alcohols for the sorption solvent improved the ability to quickly dry the complexes. Since the studied chemicals are biologically labile, the alcohol also limited the potential for any microbial contaminants to degrade the chemicals during processing. We have not determined the specific chemical nature of the interaction(s) between the chemicals and the soil constituents, but for simplicity these materials are referred to as "complexes."

Mineralization assays

Mineralization of the chemical–soil constituent complexes was performed as previously described [7]. Briefly, 0.10 g of

the soil constituent–chemical complex was added to 8.0 g (dry weight) of the soil in 50-ml serum bottles. The bottles were vortexed to distribute the complex throughout the soil, wetted to 70% gravimetric water-holding capacity (WHC) with sterile distilled, deionized water (SDDW), and then vortexed again. Controls received the chemical dosed in enough SDDW to bring the soil to 70% WHC. Four replicates were used for each treatment as well as two abiotic controls, which were prepared by autoclaving and chemical sterilization [11]. Mineralization of the ^{14}C-labeled chemicals was measured by capturing $^{14}CO_2$ on alkali-saturated wicks, as previously described [14]. Wicks were periodically removed, and radioactivity associated with the wicks was determined by liquid scintillation counting. Mineralization was measured for a period of 60 to 70 d.

Microbiological characterization

The microbial biomass of the soil, as measured by $CHCl_3$-extractable lipid phosphate, was determined using the technique of Findlay et al. [15], as previously described [7]. The physiological status of the microbial community was determined by measuring the rate of [^{14}C]acetate incorporation into total microbial lipids [16] as modified in Knaebel et al. [7].

K_d determinations

Desorption coefficients (K_d) for the montmorillonite, kaolinite, illite, sand, and HA complexes were determined using a batch desorption protocol [7,17]. Equilibria were usually reached within 12 h, but K_d determinations were measured over 48 h.

Mineralization kinetic parameter estimation

The mineralization models used in this study have been reported previously [7,18] and consisted of the first-order model [19], the 3/2-order model [20], and a zero-order model [21].

The first-order model has the form

$$P = P_0(1 - e^{-k_1 t})$$

where

P = the amount of $^{14}CO_2$ produced (%) at time t,

P_0 = the asymptotic yield (%), and

k_1 = the first-order rate constant (d^{-1}).

The mixed-order (3/2) model without a growth term has the form

$$P = P_0(1 - e^{-k_1 t}) + k_0 t$$

where

P_0 = the point at which mineralization changes from a pseudo-first-order process to a zero-order process [7], and

k_0 = the zero-order rate constant (% d^{-1}) and represents a detachment-dependent rate [7]. This is the rate of mineralization of the chemical as it becomes available from a less bioavailable pool (e.g., microbial polymers or a chemical that slowly desorbs from the soil matrix).

The zero-order model has the form

$$P = P_0 - k_0 t$$

Data were fitted to the models using the NONLIN module of SYSTAT, Evanston, Illinois, USA. The model that best fit the data was determined by the F-test procedure of Robinson

Table 1. Microbial biomass and activity of the Rossmoyne soil before and after the mineralization experiments

Soil constituent	Microbial biomass[a] (nmol lipid-PO_4 g dry wt.$^{-1}$)	Microbial activity[b] (% dpm h^{-1} g dry wt.$^{-1}$)
Initial[c]	42.2 ± 8.1	5.7 ± 0.8
Control	43.6 ± 7.1	7.9 ± 1.2
Montmorillonite	54.1 ± 5.0	11.5 ± 0.6
Kaolinite	52.2 ± 2.2	10.3 ± 1.1
Illite	51.4 ± 6.7	8.7 ± 1.2
Sand	41.0 ± 8.0	5.5 ± 0.8
Humic acids	56.0 ± 3.5	8.2 ± 1.4
Fulvic acids	51.5 ± 8.0	8.0 ± 1.2

[a] Microbial biomass (mean ± 1 SD, n = 3) measured as the amount of $CHCl_3$-extractable lipid phosphate per gram dry weight.
[b] Microbial activity (mean ± 1 SD, n = 4) determined by measuring the rate of [^{14}C]acetate incorporation into microbial lipids.
[c] Initial measurements were made on the soil prior to the addition of any of the soil constituents. Others were measured at the termination of the mineralization experiments.

[22]. Statistical analyses were performed using the GLM module of SAS, Cary, North Carolina, USA, and the MGLH module of SYSTAT. Comparisons within treatments were done using the Scheffé test [23].

RESULTS

This Rossmoyne soil is classified as a fine-silty mixed Mesic Aquic Fragiudalf [8]. It had a higher relative abundance of hydroxy–Al interlayered vermiculite (or smectite) than smectite, more smectite than vermiculite, and no detectable mica. The soil was acidic (pH = 4.5 ± 0.3) and relatively low in organic carbon (0.9%) and had a cation exchange capacity of 19.8 ± 0.8 meq 100 g^{-1} and a surface area of 20.4 m^2 g^{-1}.

The microbial biomass of the Rossmoyne soil changed during the course of the experiments, but the soil constituent treatments were not significantly different from the control treatments (Table 1) ($p > 0.050$, Scheffé test). The physiological activity of the soil-constituent-amended soils also changed during the course of the experiments (Table 1). The montmorillonite- and kaolinite-treated soils had elevated activities in comparison to the initial activity; the sand treatment had a slightly depressed activity. These were significantly different from each other ($p < 0.05$) but not from the control treatment ($p > 0.05$).

The microbial mineralization of the different forms of the different chemicals showed interesting patterns that could be attributed to the influence of the soil constituent and to the parent chemical (Figs. 2 to 4, Table 2). However, it should be noted that when the chemicals were dosed to the soil in water, they were mineralized immediately (no acclimation periods were observed). For all the sorbed chemicals studied, the sand-, montmorillonite-, and illite-bound forms of the chemicals were mineralized at the greatest initial rates ($p < 0.05$, Scheffé test). The kaolinite-bound forms were metabolized slightly less rapidly than the sand-bound forms ($p < 0.05$) but similarly to the illite-bound forms. When the chemicals had been bound to HAs or FAs, they were mineralized at significantly slower initial rates than all other forms. The soil constituents also had relatively consistent effects on the extents of mineralization. The kaolinite- and sand-bound forms and control additions were mineralized to the greatest final extents; the illite was mineralized less than these forms, followed by montmorillonite-bound forms and the FA-bound forms. Some of the chem-
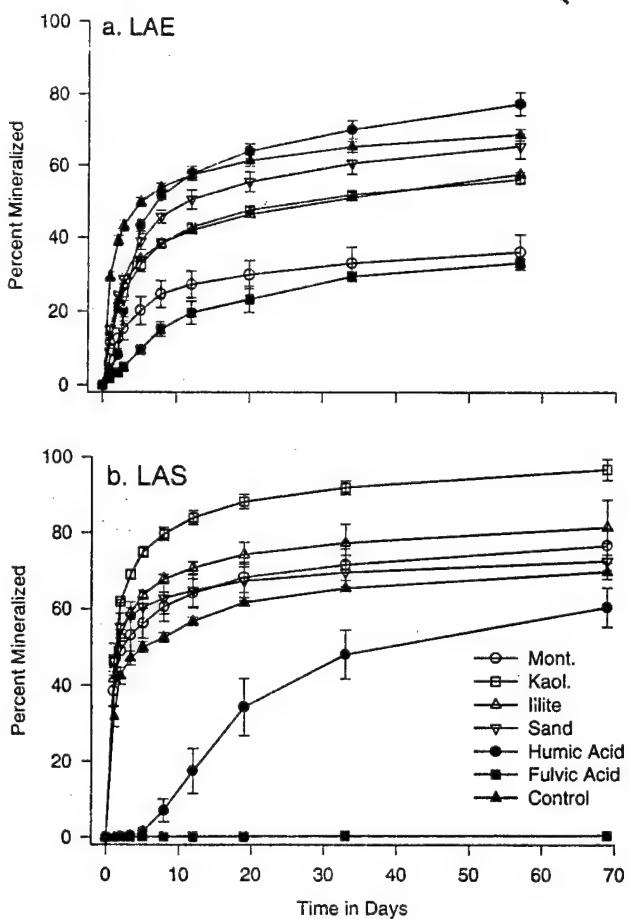
Fig. 2. (a) Percent production of $^{14}CO_2$ from the mineralization of LAE that had been adsorbed to the different soil constituents or added to the Rossmoyne soil in an aqueous carrier. Error bars indicate 1 SD ($n = 4$). (b) Percent production of $^{14}CO_2$ from the mineralization of LAS that had been adsorbed to the different soil constituents or added to the Rossmoyne soil in an aqueous carrier. Error bars indicate 1 SD ($n = 4$). The soil constituents to which the surfactants were bound are shown in panel b.

icals that had been bound to HAs were mineralized to great extents (LAE and LAS), but others were not (stearate, STAC, and C_{12}TMAC). It was also apparent that the chemicals had significant influence on the observed mineralization patterns, which are described below.

Dodecyl linear alcohol ethoxylate was mineralized immediately and to generally great extents (Fig. 2a) and was mineralized differently as a function of the soil constituent to which it was bound. When LAE was added to the soil in water alone, it was mineralized at an initial rate that was almost twice as fast as the mineral-bound forms (montmorillonite, kaolinite, illite, and sand) ($k_1 = 0.68$ d^{-1} vs. 0.33 ± 0.5 d^{-1}) and four times faster than when it was bound to HAs or FAs ($k_1 = 0.15$ and 0.09 d^{-1}, respectively). All the mineral-bound forms were mineralized at similar initial rates (0.34 ± 0.05 d^{-1}), and three of these (sand-, illite-, and kaolinite-bound forms) were also mineralized to similar extents ($P_0 = 42.4 \pm 5.1$; final % $= 59.0 \pm 3.8$). However, the montmorillonite-bound LAE was mineralized to substantially lesser extents (final % $= 36$). The HA-bound LAE was eventually mineralized to extents greater than all other forms of LAE, whereas FA-bound LAE was mineralized to lower extents than all other forms.

The mineralization of the different forms of LAS differed considerably from LAE (and from the other chemicals). All mineral-bound forms and the control were immediately mineralized at high rates ($k_1 = 0.88 \pm 0.21$ d^{-1}) and to relatively large extents ($P_0 = 64.6 \pm 9.8\%$). In contrast to the mineralization of LAE (and the other chemicals), the control form of LAS was mineralized more slowly and to lesser extents than the mineral-bound forms (Fig. 2b, Table 2). The mineralization of the HA-bound LAS did not begin until after a 5-d lag period, whereas no mineralization of the FA-bound LAS was observed. After the mineralization of HA-bound LAS began, it was mineralized at a rate approximately half as fast as the other mineral-bound or control forms (Table 2).

The different forms of stearate were all mineralized immediately but at slower rates and to lesser extents than LAE or LAS (Fig. 3). In addition, the different forms had substantially different initial rates (k_1), P_0 estimates, and final yields (Fig. 3, Table 2). The k_1 estimates of mineralization of the different forms ranged from very slow (0.09 d^{-1} for FA-bound stearate) to relatively fast (0.41 d^{-1} for kaolinite- and sand-bound stearate). The control form of stearate was mineralized only slightly slower than these two forms, followed by the illite- and montmorillonite-bound forms. The HA-bound form was mineralized more slowly than the illite-bound form but to similar extents. The FA-bound stearate complex was initially mineralized more slowly than all other forms ($k_1 = 0.09 \pm 0.02$ d^{-1}) and was also mineralized to lesser extents than the other forms (36% vs. an average of 49%). Its k_0, however, was comparable to that of the other forms (0.27 ± 0.10 vs. an average of 0.30 ± 0.06).

The two cationic chemicals, STAC and C_{12}TMAC, were mineralized at slower rates and to much lower extents than the other chemicals (Table 2, column averages). The initial rates of mineralization of all forms of STAC were greater than those for C_{12}TMAC (Table 2, $k_1 = 0.33 \pm 0.11$ for STAC vs. 0.08 ± 0.01 for C_{12}TMAC) and similar to the initial rates of LAE mineralization (Table 2). However, the k_1 estimates for STAC mineralization should be evaluated with the other parameter estimates in mind, since k_1 represented the initial rate of mineralization of only 4.5% of the total STAC added to the soil (Table 2, column average for STAC P_0). In comparison, the initial rates for LAE mineralization reflected the mineralization of a much larger fraction of the total LAE ($42.8 \pm 13.9\%$, Table 2). Except for the initial quickly mineralized pool of STAC, its mineralization resembled the C_{12}TMAC mineralization (Fig. 4a and b). However, the different forms of C_{12}TMAC had faster k_0 rates of mineralization and were also mineralized to greater extents than the same forms of STAC. However, some forms of C_{12}TMAC (montmorillonite- and FA-bound) were not mineralized to any significant degree.

The 3/2-order mineralization model provided some unique parameters for evaluating different patterns of mineralization. These unique parameters, k_1, P_0, and k_0, allowed us to discriminate between an initially bioavailable pool of a chemical (defined by k_1 and P_0) and a less bioavailable pool (defined by P_0 and k_0). The point at which mineralization of a chemical changes from a first-order process to a zero-order process occurs at P_0 [7]. The greater the value of P_0, the greater the amount of the chemical that is immediately available for microbial biodegradation. The rate of degradation of this pool was provided by k_1; the remainder of the chemical is degraded more slowly, and this rate is provided by k_0. We observed that these parameters were a function of the chemical itself and

Table 2. Mineralization kinetic estimates[a] for the biodegradation of the adsorbed surfactants in the Rossmoyne soil

Values are given as mean (± 1 SD).

Soil constituent	LAE				LAS				Na stearate				STAC				C₁₂TMAC			
	k_1	k_0	P_0	Final	k_1	k_0	P_0	Final	k_1	k_0	P_0	Final	k_1	k_0	P_0	Final	k_0	k_1	P_0	Final
Montmorillonite	0.30 (0.09)	0.17 (0.04)	26.8 (4.3)	36.2 (4.9)	0.87 (0.11)	0.29 (0.30)	59.0 (3.5)	70.2 (4.2)	0.14 (0.05)	0.18 (0.09)	26.4 (3.4)	38.6 (3.3)	0.51 (0.28)	0.13 (0.13)	3.4 (0.2)	12.2 (4.7)	c			2.1 (0.1)
Kaolinite	0.31 (0.03)	0.28 (0.01)	40.9 (0.7)	56.2 (0.9)	0.74 (0.06)	0.25 (0.12)	78.8 (1.9)	96.2 (2.8)	0.40 (0.02)	0.35 (0.01)	32.6 (1.1)	58.5 (1.3)	0.24 (0.03)	0.20 (0.05)	5.2 (0.7)	18.9 (3.1)	0.20 (0.03)	0.07 (0.02)	10.1 (3.1)	21.8 (0.9)
Illite	0.40 (0.06)	0.35 (0.01)	38.3 (0.7)	57.5 (0.7)	0.82 (0.11)	0.25 (0.12)	66.4 (1.5)	81.3 (7.3)	0.31 (0.01)	0.30 (0.01)	25.2 (0.5)	47.6 (0.9)	0.35 (0.04)	0.09 (0.01)	5.1 (0.9)	11.5 (0.7)	0.21 (0.02)	0.07 (0.01)	6.8 (1.4)	18.3 (1.6)
Sand	0.31 (0.02)	0.32 (0.03)	48.1 (2.5)	63.4 (4.6)	1.25 (0.19)	0.19 (0.02)	66.2 (4.0)	72.8 (4.7)	0.41 (0.01)	0.34 (0.05)	38.0 (1.3)	62.0 (2.7)	0.33 (0.09)	0.14 (0.02)	5.9 (0.9)	13.2 (2.0)	0.18 (0.03)	0.09 (0.02)	8.0 (1.1)	18.6 (0.8)
Humic acids	0.15 (0.01)	0.19 (0.04)	65.8 (1.9)	77.4 (3.2)	0.41 (0.01)	d / f	67.3 (5.1)	60.4 (5.9)	0.19 (0.01)	0.36 (0.01)	28.5 (1.8)	45.2 (1.6)	0.36 (0.06)	0.11 (0.03)	3.6 (1.0)	5.2 (0.4)	0.19 (0.02)	c	1.1	10.1 (0.9)
Fulvic acids	0.09 (0.01)	0.11 (0.12)	27.4 (8.1)	33.4 (1.1)					0.09 (0.02)	0.27 (0.10)	20.0 (4.2)	35.8 (2.9)	0.16 (0.02)	0.04 (0.01)	2.8 (0.4)	4.7 (0.4)	c			0.9 (0.1)
Control[b]	0.68 (0.03)	0.33 (0.01)	52.0 (1.2)	68.8 (1.6)	0.74 (0.10)	0.29 (0.01)	52.4 (0.9)	69.7 (0.5)	0.36 (0.02)	0.31 (0.02)	35.8 (0.3)	55.2 (1.0)	0.4 (0.04)	0.13 (0.05)	5.6 (0.4)	13.2 (0.6)	0.23 (0.06)	0.07 (0.02)	9.3 (2.7)	21.7 (2.0)
Mean[g]	0.32 (0.19)	0.25 (0.09)	42.8 (13.9)	56.1 (16.2)	0.80 (0.30)	0.25 (0.04)	65.0 (8.9)	75.1 (12.3)	0.27 (0.13)	0.30 (0.06)	29.5 (6.3)	49.0 (10.0)	0.33 (0.11)	0.12 (0.05)	4.5 (1.2)	11.3 (4.9)	0.20 (0.02)	0.08 (0.01)	8.6 (1.5)	13.4 (9.0)

[a] Estimates are the means (top line) ± 1 SD (bottom line), $n = 4$.
[b] Added to soil in aqueous carrier.
[c] Kinetic parameters not calculable because of insubstantial mineralization.
[d] These LAS data were best fit by a first-order model with a lag time of 5.2 d prior to the onset of mineralization.
[e] These C₁₂TMAC data were best fit by a zero-order model; k_0 is the percent mineralized per day.
[f] No mineralization detected.
[g] Mean row average and standard deviations calculated from the mean value of each treatment.
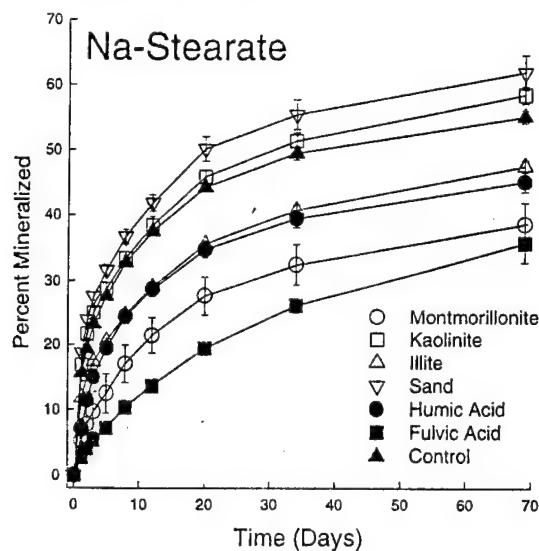
Fig. 3. Percent production of $^{14}CO_2$ from the mineralization of Na stearate that had been adsorbed to the different soil constituents or added to the Rossmoyne soil in an aqueous carrier. Error bars indicate 1 SD ($n = 4$).
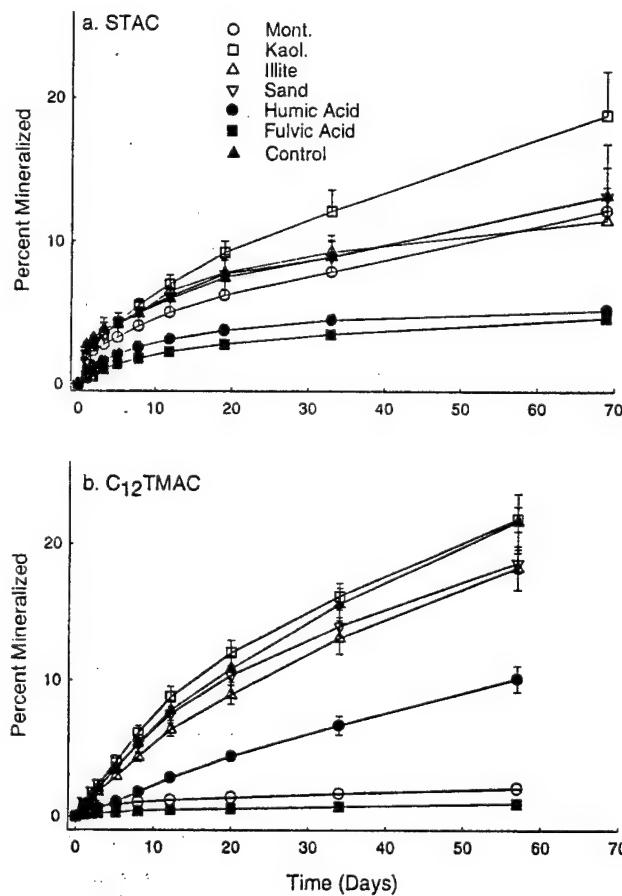

Fig. 4. (a) Percent production of $^{14}CO_2$ from the mineralization of STAC that had been adsorbed to the different soil constituents or added to the Rossmoyne soil in an aqueous carrier. Error bars indicate 1 SD ($n = 4$). (b) Percent production of $^{14}CO_2$ from the mineralization of $C_{12}TMAC$ that had been adsorbed to the different soil constituents or added to the Rossmoyne soil in an aqueous carrier. Error bars indicate 1 SD ($n = 4$).

the soil constituent to which it was bound. When examining the control treatments, LAS and LAE were initially the most bioavailable chemicals ($P_0 = 52\%$), stearate was intermediate (36%), and STAC and $C_{12}TMAC$ were less bioavailable (6 and 9%, respectively).

The soil constituents had substantial effects on these mineralization parameters and therefore on the bioavailability of the chemicals. When rank-ordered over all the chemicals, the sand-bound forms were the most available to the first-order processes, followed by the control treatment, then the montmorillonite-, kaolinite-, and illite-bound forms. The organic matter (HA and FA)-bound chemicals were considered apart from the mineral forms because they behaved differently (Figs. 2a and 4a) or were not mineralized at all (Figs. 2b and 4b). For the HA-bound forms that could be fitted to the 3/2-order model, the pool that was mineralized in a first-order fashion was relatively large. However, for the FA-bound chemicals, the P_0 values were usually the lowest observed.

The mineral forms of most of the chemicals had striking similarities in their k_0 rates. The control and all mineral-bound forms of LAS had similar k_0 rates ($0.25 \pm 0.04\%$ d^{-1}). The control treatment and the mineral forms (excluding the montmorillonite-bound form) of LAE were also mineralized at similar rates ($k_0 = 0.32 \pm 0.03\%$ d^{-1}), as were the same forms of stearate ($k_0 = 0.33 \pm 0.02\%$ d^{-1}) and $C_{12}TMAC$ ($k_0 = 0.21 \pm 0.02\%$ d^{-1}). In contrast, the k_0 rates of the different forms of STAC followed no trend of this type.

The desorption coefficients (K_d) also provided information regarding the bioavailability of the different forms of these chemicals. The anionic and nonionic chemicals were generally less tightly sorbed to the soil constituents than the cationic chemicals (Table 3). Dodecyl linear alcohol ethoxylate and LAS had the lowest log K_d values (average of ~2.1), whereas stearate was higher (2.73) and $C_{12}TMAC$ and STAC were the highest (3.41 and 3.03, respectively). The sorptive behaviors of the chemicals on the different soil constituents were very uniform for some chemicals (LAE), whereas others showed more variability (LAS and stearate). The quaternary ammonium compounds ($C_{12}TMAC$ and STAC) had intermediate variability.

When these desorption coefficients were compared to the mineralization kinetic parameter estimates (Table 2), some strong relationships were apparent (Fig. 5). The initial rate of mineralization, k_1, was negatively correlated with K_d ($r = -0.669$), as were the yield estimate, P_0, ($r = -0.668$) and the final percent recovery of $^{14}CO_2$ ($r = -0.708$). The detachment-dependent rate, k_0, however, was not correlated with K_d ($r = -0.209$). The relationships between the mineralization parameters and K_d values were influenced by the different chemicals more than by the soil constituents (Fig. 5). For example, LAS and LAE were mineralized to the highest amounts and at the greatest rates, and most forms had low K_d values. In contrast, the different forms of STAC and $C_{12}TMAC$ were mineralized less and had much larger K_d values than the other surfactants. The different forms of stearate had intermediate K_d values and were mineralized at intermediate levels. The soil constituents had less influence on the relationships between the mineralization parameters and K_d values. The soil constituents did not significantly affect the slope of the regression but were distributed along it as a function of the chemical to which they were adsorbed (data not shown).

The FA-bound chemicals were omitted from the correlation analyses because of problems in determination of their K_d

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Table 3. Desorption coefficients, log K_d,[a] for the surfactant–soil constituent complexes[b]

Soil constituent	Chemical				
	LAE	LAS	Na Stearate	C_{12}TMAC	STAC
Sand	1.99	1.61	2.68	3.00	2.90
	0.15	0.18	0.47	0.64	0.90
Kaolinite	2.21	2.19	3.15	3.32	3.37
	0.09	0.31	0.14	0.22	0.06
Illite	2.15	1.82	2.83	3.72	3.10
	0.09	0.13	0.54	0.60	0.84
Montmorillonite	1.99	1.34	1.52	3.30	2.43
	0.17	0.15	0.17	0.26	0.20
Humic acids	2.13	3.23	3.46	3.70	3.35
	0.41	0.29	0.41	0.87	1.21
Mean of average ± SD	2.09 ± 0.10	2.04 ± 0.74	2.73 ± 0.74	3.41 ± 0.30	3.03 ± 0.39

[a] K_d calculated as $(G_s/B)/C_s$, where G_s = total quantity of material desorbed (μg), B = dry weight of soil (or soil constituent), and C_s = concentration of chemical in solution at equilibrium (μg mL^{-1}). Due to difficulty in determining accurate K_d values for the fulvic acid complexes, these data were omitted from this table. Estimates are the mean (top line) ± 1 SD (bottom line), $n = 3$.
[b] Some data adapted from Knaebel et al. [7].

values. Since the FA-bound chemical complexes were water soluble at any pH, a dialysis-based system was used to determine the K_d values. However, due to kinetic limitations and interactions between the chemical and the dialysis tubing, the derived K_d estimates were very low or appeared to be a function of chemical molecular weight, not desorption (data not shown).
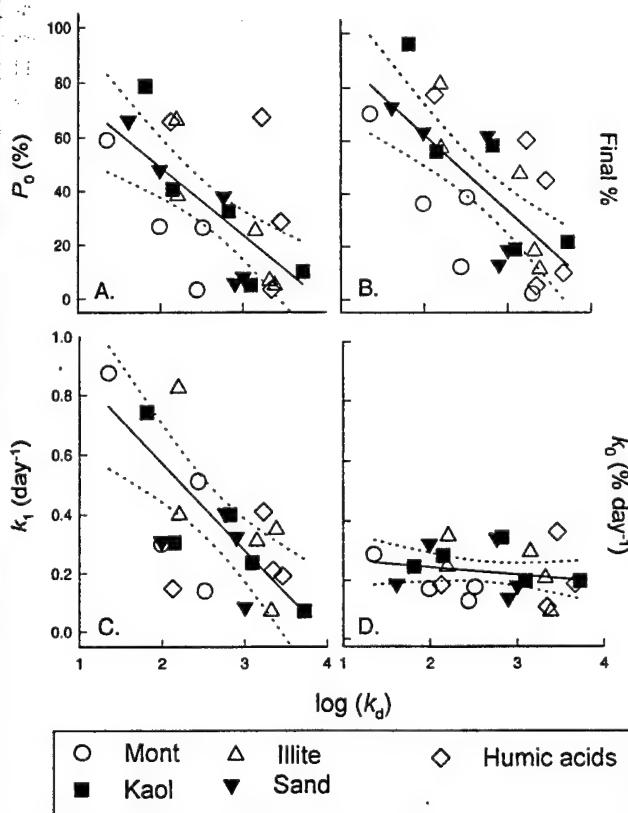
Fig. 5. Relationship between the log of the desorption coefficient, K_d, and the mineralization kinetic parameter estimates (95% confidence intervals are shown). (A) Log K_d versus P_0. (B) Log K_d versus final percent recovered $^{14}CO_2$. (C) Log K_d versus k_1. (D) Log K_d versus k_0.

DISCUSSION

The present study demonstrated that the mineralization of a chemical by soil microbial communities is dependent on the nature of the chemical and the fraction of the soil with which it interacts prior to biodegradation. The chemicals themselves differed widely in their mineralization kinetics, from some that were quickly degraded to high extents (LAE and LAS) to those that were slowly mineralized to lower extents (STAC and C_{12}TMAC). The soil constituents, however, also had substantial influence on the chemical's degradation. If the chemicals were first bound to some soil constituents (e.g., kaolinite or sand), they were quickly mineralized at rates and extents equivalent to when they were added to the soil in aqueous solutions. However, when the chemicals were first bound to other soil components (e.g., montmorillonite, HAs, or FAs) their mineralization was almost always slower or delayed. Usually, interactions with these soil constituents inhibited degradation, but in one case (i.e., LAE interaction with HAs), the eventual mineralization was slightly greater than in the control treatments.

The chemical–soil constituent complexes were degraded similarly in the woodlot soil [7], but there was a wider variability in the agricultural soil, potentially because of its more weathered and acidic nature (Table 4).

Mineralization model

The 3/2-order mineralization model fits mineralization data more closely than a first-order model [7]. We found that this model provided insight into the fate of a chemical in a soil environment. Specifically, the parameters permitted discrimination of bioavailable pools of a chemical from those pools that are less bioavailable.

The initial rate constant estimate (k_1) represents the rate of mineralization of the fraction of the chemical that is readily available to the microbial community [7]. The size of this pool is provided by the P_0 estimate. The rate of mineralization of the less bioavailable pool is estimated by a zero-order rate estimate (k_0) that indicates the rate of mineralization of a chemical as it slowly becomes available from either the soil matrix and/or from microbial storage pools [7,20].

Table 4. Soil characteristics of the Bonnell and Rossmoyne soils

Soil characteristic	Bonnell	Rossmoyne
pH	7.1	4.5
Organic carbon (%)	4.7	0.9
Cation exchange capacity (meq 100 g^{-1})	27.5	19.8
Surface area (m^2 g^{-1})	49.6	20.4
Clay mineralogy[a]		
Mica	++++	
Vermiculite	+++	+
Smectite	+	+++
OH–Al interlayered vermiculite or smectite		++++

[a] Relative abundance: ++++ = major; +++ = moderate; and + = trace.

The k_0 term as presented by Brunner and Focht [20] represented the indigenous rate of C turnover in a soil. Previously, we suggested that it represents the mineralization of either the parent chemical or metabolites that slowly become available from the soil matrix. The present study permitted further analysis of k_0 and strengthens the argument that it reflects the mineralization of the pool of the chemical that has become associated with the parent soil matrix. This is because k_0 was relatively constant for the chemicals which had been bound to less reactive soil minerals or when added to the soils in aqueous solution. The only way that a consistent pool of chemical could be formed in these treatments was if most of the chemical desorbed from the soil constituent to which it was originally bound. Part of this pool became available for microbial uptake and was degraded at a rate estimated by k_1. The other desorbed fraction became associated with the parent soil matrix and was mineralized at a rate estimated by k_0. As this latter pool became available for microbial mineralization, the rate would be identical for the chemical, regardless of the original soil constituent to which it was bound, since it is now dependent on the chemical's interaction with the parent soil matrix. This conclusion was also supported by the experimental design used, where a small relative amount of the chemical–soil constituent complex was added to the soil. Thus, desorption of the chemical from the original complex would be followed by either microbial uptake or interactions with the parent soil matrix.

At this time, we cannot discern the nature of this interaction or with what soil fraction it occurs (microbial, soil minerals, or soil organic matter). We observed that it was chemical specific and that it changed from soil to soil [7]. Therefore, if a chemical enters a soil on a relatively small amount of a nonreactive carrier and is well dispersed, once it desorbs from the carrier (and the initially available pool is degraded), its secondary rate of mineralization will be determined by the soil matrix, not by the carrier.

Influence of the different soil constituents on biodegradation

The mineralization kinetics of the different forms of these chemicals yielded substantial information as to the mechanisms that control the bioavailability of the chemicals to the soil microbial community. These experiments provided further evidence that interactions with the relatively nonreactive mineral surfaces of sand, kaolinite, and to some degree illite have little effect on the microbial utilization of the chemicals [7]. Once these complexes were distributed to the soil and wetted

to environmentally realistic levels, the chemicals were freely available to the microbial community or became associated with the parent soil matrix. Although the initial rates of mineralization and overall amounts mineralized varied from chemical to chemical, these mineral forms were essentially as bioavailable to the microbial community as when the chemical was distributed to the soil in an aqueous solution. Support for this bioavailability is demonstrated by the uniformity of the k_0 rate estimates for these forms of each chemical as well as their high and consistent P_0 estimates. On the other hand, the more reactive montmorillonite, HAs and FAs inhibited not only the initial rates and extents of mineralization but also the detachment-dependent rates (k_0).

Significant relationships were observed between the desorption of the chemicals from the different soil constituents (as estimated by K_d) and their mineralization kinetic estimates (Fig. 5). We found that k_1, P_0, and the final percent were all negatively correlated with K_d, whereas k_0 exhibited little correlation with K_d. These relationships appeared to be defined mostly by the inherent sorptive nature of the chemical rather than the differences in the different soil constituents [7]. This was somewhat unexpected, but when considering the different chemicals studied, it would be expected that differently charged molecules would behave differently with these soil minerals or organic materials. The differences between the chemicals of similar type but with different alkyl chain lengths suggests that for anionic compounds (LAS and stearate), the longer alkyl portion results in higher K_d values, but for cationic compounds (C$_{12}$TMAC and STAC), a longer alkyl moiety tends to decrease the K_d value (Table 3). These differences can be attributed to hydrophobic interactions of the longer alkyl chain overriding the repulsive forces for the anionic compounds and the longer alkyl moieties inhibiting ionic attractions for the cationic compounds. These interpretations should be considered in light of the variability associated with individual K_d determinations. Some of the chemical–soil constituent complexes showed greater within-treatment variability (e.g., HA complexes), whereas others were less variable (e.g., montmorillonite complexes). Likewise, some of the chemicals also had wider variabilities (e.g., STAC) than others (e.g., LAE).

Some of these soil minerals have shown similar effects on the fate of other xenobiotic chemicals but not in systems that used a native soil community as a test matrix. Kaolinite had no significant effects on the biodegradation of diquat in nutrient solutions, whereas montmorillonite inhibited its degradation [24]. In another artificial system, nucleic acids were degraded less in the presence of montmorillonite than in the presence of kaolinite or illite, as measured by O_2 uptake [5]. The present experiments confirmed that interactions with montmorillonite tended to lessen the immediate and long-term bioavailability of the cationic and nonionic surfactants but had little effect on the anionic LAS. This phenomenon was attributed to the aromatic nucleus of LAS being too large (~35 Å) to enter the interlayers of an expanded montmorillonite clay (~10–20 Å) [7], as was observed for 2,4-dichlorophenoxyacetic acid sorption and biodegradation in the presence of montmorillonite [25]. Another chemical with an aromatic nucleus (alachlor) was also unable to enter the interlayers of montmorillonite when the clay had been saturated with monovalent cations [26]. Most of the other chemicals, although of equal or greater mass, did not have a moiety with a radius as large as the aromatic ring and therefore could partition into

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the interlayer spaces, which would result in a pool of the chemical that would be unavailable for microbial degradation. Further support for this size exclusion phenomenon is provided by the behavior of the cationic chemicals $C_{12}TMAC$ and STAC in the presence of the montmorillonite. In almost every other environmental form, STAC was mineralized less than $C_{12}TMAC$ (Table 2). However, montmorillonite-bound STAC was mineralized more quickly and to greater extents than montmorillonite-bound $C_{12}TMAC$. The only way that STAC would have been more available to microbial degradation than the smaller $C_{12}TMAC$ was if the former was not able to enter the interlayers of the montmorillonite.

The HA- and FA-associated chemicals were usually mineralized more slowly and to lesser extents than the other forms, with FA-associated chemicals being less degraded. This was a common trend (Figs. 2a and b, 3, and 4a and b), but the anionic LAS associated with HA was only degraded after a ~5-d lag period, and the LAS associated with FA was not observably degraded during the course of this experiment (Fig. 2b). A notable exception to this trend was when LAE was associated with HA and was eventually mineralized to equivalent or greater extents than any other form (Fig. 2a). Except for this one case, this study confirms other studies that found inhibitory effects of soil organic matter components on the utilization of a wide array of chemicals [5,27–31]. The unique contribution of the present study is that it suggests that different soil organic matter pools have different effects on the biodegradation of a chemical.

The FAs often rendered the chemicals less degradable than HAs, generally by a factor of 2. This was unexpected, as HAs are usually thought to offer a greater barrier to microbial access than FAs, in part because HAs are orders of magnitude larger than FAs [32]. In particular, HAs contain longer-chain fatty acid products than FAs, suggesting that HAs are also more hydrophobic than FAs [33]. Since the FAs and HAs were extracted from different parent materials, the differences in their observed effects could have been a result of these material differences rather than their different chemical natures. In addition, even though the FAs were twice purified by anion exchange chromatography, there were significant amounts of residual sodium chloride, which has been shown to be a common contaminant [32], in the material. This salt may also have had an effect on the degradation of the chemicals complexed with the FAs. We plan further studies to investigate the relationships between the source of the HAs and FAs, different extraction techniques, and the effects of these on sorption, bioavailability, and biodegradation.

Influence of the parent soil on biodegradation

We have noted previously that overall soil microbial biomass and activity were not correlated with the mineralization of some chemicals in a natural woodlot soil (Bonnell) [7]. We also found no relationship between the microbial biomass and activity and mineralization kinetics of these chemical–soil constituent complexes in the Rossmoyne soil. This suggests that the mineralization of these chemicals at the concentrations studied is not dependent on community-level microbial characteristics but is due to efficient uptake and degradation of the chemical by ubiquitous populations that are not discernible by these community-level assays. It also suggests that other soil factors may control the bioavailability, and therefore the biodegradation, of these chemicals.

From a pedogenic perspective, the Rossmoyne soil used in

this study was more weathered than the Bonnell soil [7] studied previously (Table 4). The nature of this soil suggests that most chemical interactions would occur on the mineral surfaces and not on humic coatings on the minerals, as was suggested for the Bonnell soil. These interactions would be primarily hydrogen bonding and ligand exchange reactions, whereas the Bonnell soil would have been dominated by hydrophobic bonding and metal bridging. These characteristics may explain some of the mineralization differences of these chemicals in the Rossmoyne soil as compared to the Bonnell soil and help elucidate mechanisms that control the bioavailability of the chemicals.

All forms of the anionic LAS except the FA-bound form were mineralized at faster initial rates and to greater extents in the Rossmoyne soil than in the Bonnell soil [7]. It is likely that the low amount of organic matter coatings on the mineral surfaces resulted in fewer hydrophobic interactions between the alkyl moiety and/or the aromatic nucleus of LAS and similar moieties in the soil organic matter matrix. Therefore, relatively more LAS would be present in the Rossmoyne soil solution which would have been available for microbial uptake [25]. The greater degradation of LAS in the Rossmoyne soil could not be attributed to lower soil pH, since this would have tended to result in more sorption of the anionic LAS to the soil mineral surfaces and lowered its overall mineralization. This would be especially true if there were little or no Al- or Fe-oxide coatings on the surfaces of the Rossmoyne soil. However, these oxide determinations were not measured for this soil or the Bonnell soil.

The lack of mineralization of the FA-bound LAS suggests that the hydrophobic and/or ionic interactions between LAS and this more ionic soil organic matter fraction were stable enough to prohibit it from becoming available to the microbial community. The differences in LAS mineralization complexed with FA may have been due to the greater amount of carboxyl residues in the FAs than in the HAs [33]. These may have resulted in more cationic bridging between the carboxyl residues of the FAs and the sulfonate group of LAS, rendering it unavailable for degradation.

The different forms of the anionic stearate were also mineralized at faster initial and secondary rates and to greater extents in the Rossmoyne soil as compared to the Bonnell soil [7]. This strengthens the argument that it was the lack of hydrophobic coatings in the Rossmoyne soil and not pH effects that improved the anionic chemical degradation. Furthermore, the pH of this soil would be low enough to permit some formation of the stearic acid (pK_a ~4.85), which would be less soluble than the sodium salt. If soil organic matter had been more prevalent in this soil, the stearic acid would likely have partitioned into it and been less available to the microbial community. Since the mineral and control forms of stearate were mineralized to substantial amounts, it is not likely that a significant fraction partitioned into the native soil organic material. However, when both the FA and HA forms were added to the Rossmoyne soil, the stearate was mineralized much more slowly and to lesser extents.

Further support for the nature of the mineral surface controlling the mineralization of the different forms of the chemical is supplied from the mineralization of the different forms of the cationic chemicals, STAC and $C_{12}TMAC$. The different forms of STAC were mineralized more in the Bonnell soil than in the Rossmoyne soil [7]. In another study, $C_{12}TMAC$ was also mineralized more quickly and to greater extents in the

Bonnell soil compared to the Rossmoyne soil [34]. In the present study, all forms of $C_{12}TMAC$ were mineralized at faster secondary rates and to greater extents than STAC. The charge to mass ratio of $C_{12}TMAC$ is greater than that of STAC, owing to STAC having an alkyl chain that is six carbons longer than that of $C_{12}TMAC$. In contrast, hydrophobic interactions would be stronger for STAC than $C_{12}TMAC$. These characteristics led us to predict that $C_{12}TMAC$ would be degraded to greater extents in an acidic soil due to greater repulsion of $C_{12}TMAC$ from the positively charged soil surfaces. The characteristics also led us to predict that soils with higher organic matter content would result in less mineralization of the more hydrophobic STAC.

Experimental evidence demonstrated that both STAC and $C_{12}TMAC$ were degraded more in a neutral pH, organic soil than in an acidic, mineral soil [7,34] and that $C_{12}TMAC$ was degraded to slightly greater extents than STAC. Although these chemicals have not been studied in an wide range of soil types, the trend suggests that the differences were due to relative amounts of different clay types present in the soils (Table 4) rather than pH or hydrophobic interactions. Both chemicals were degraded more in the less weathered Bonnell soil, which did not contain a large amount of substituted or unsubstituted smectites but did contain relatively more mica. The greater abundance of weathered clays in the Rossmoyne soil would permit more cation exchange reactions, which would presumably result in less availability of either of the cationic chemicals.

In contrast to both LAS and stearate, the nonionic chemical LAE was mineralized in the Rossmoyne soil at almost identical levels as in the Bonnell soil. This suggests that the different natures of the soil matrix had less effect on the bioavailability of this nonionic chemical and that it remained relatively bioavailable to the microbial community. This strengthens the argument that cation exchange limited the degradation of the cationic chemicals and that cationic bridging limited the degradation of the anionic surfactants. The nonionic LAE would not be as susceptible to these interactions and so was relatively available and degradable in both soils.

CONCLUSIONS

This study demonstrated the complex interactions that occur when different chemicals interact with specific fractions of soil and are subjected to the overall soil chemistry. It supports a concept that we have observed previously [7]: The nature of the chemical itself has a high degree of influence on its bioavailability and subsequent biodegradation. It demonstrates that if a chemical interacts with a specific soil component prior to biodegradation, the initial interaction may have little effect on its subsequent biodegradation, if the interaction is with nonreactive minerals. In contrast, interactions with more reactive minerals (montmorillonite) or soil organic matter may have tremendous effects on the microbial degradation of the chemical. Usually, these soil components had similar effects (either stimulatory or inhibitory) on the different chemicals that we studied. However, there were some notable exceptions (LAS not being negatively affected by interactions with montmorillonite and LAE not being inhibited from long-term mineralization when associated with HAs). By examining the fate of these preadsorbed chemicals in a natural and agricultural soil, we were able to discriminate the magnitude of these effects in a natural system without massive changes in soil chemistry, mineralogy, or microbiology.

Chemicals of the type studied (surfactants) usually enter soils in association with sewage sludges that are spread as agricultural soil amendments. Other chemical (pesticides, fertilizers, and pollutants) also commonly enter soils in association with a chemically inert carrier or in an undefined complex matrix. This applied form of the chemical adds another level of complexity to understanding the fate of chemicals in soils. We plan to investigate the fate of the carrier–chemical complex association to better understand and predict how the chemical, its carrier, and the components of the soil matrix interact and affect the ultimate fate of chemicals in soils.

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OXYGEN LIMITATIONS AND AGING AS EXPLANATIONS FOR THE FIELD PERSISTENCE OF NAPHTHALENE IN COAL TAR-CONTAMINATED SURFACE SEDIMENTS

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Abstract—Naphthalene has been transported approx. 400 m via groundwater flow from buried subsurface coal tar to an organic matter-rich seep area where the water emerges at the foot of a hill in a field study site. We have tested several hypotheses for explaining why naphthalene persists in seep sediments. In aerobic laboratory flask assays, conversion of ^{14}C-naphthalene to $^{14}CO_2$ occurred and was not stimulated by amendments with vitamins or inorganic nutrients. Thus, neither toxicity nor nutrient limitation were the cause of naphthalene persistence. At the site, in situ sediment oxygen concentrations were below detection. Oxygen-limited naphthalene biodegradation was demonstrated both by measuring no conversion of ^{14}C-naphthalene to $^{14}CO_2$ in samples of seep sediments prepared anaerobically and by measuring naphthalene loss from anaerobic nitrate-amended slurry-phase incubations of the sediment only after O_2 was added. However, when H_2O_2 was added as an O_2 source to site sediments in situ in a randomized block design, no discernible naphthalene loss occurred. The possibility that decreased bioavailability might contribute to naphthalene persistence was investigated by monitoring $^{14}CO_2$ evolved by microorganisms added to γ-ray sterilized sediments that had been exposed under aseptic conditions to ^{14}C-labeled naphthalene for periods ranging from 0 to 28 d. Resulting patterns in the extent and rate of naphthalene mineralization revealed an inverse relationship to the duration of contact with the sediment, but only when the mixed microbial inoculum had been enriched on aqueous-phase naphthalene. We conclude that oxygen limitation is the most probable cause for lack of naphthalene biodegradation at our field study site. However, diffusion or sorption reactions may also play a role.

Keywords—Naphthalene   Biodegradation   Field site   Sorption   Oxygen limitation

INTRODUCTION

The persistence of organic contaminant compounds in field sites can pose serious health hazards to both wildlife and humans that may be exposed to site-derived waters, soils, or sediments [1–3]. Conversely, in situ microbial activity in field sites has the potential to mineralize contaminant organic compounds, thereby eliminating toxicological risks. For several years we have been examining the fate of coal tar-derived polycyclic aromatic hydrocarbons (PAHs) at a field study site in upstate New York [4–6]. Past and ongoing measurements using solvent extraction and gas chromatography procedures [7–9] indicate that PAH compounds at the field site occur in a groundwater plume that extends from a point source (a coal tar waste disposal pit) to a seep area, where ground water and aquifer sediments are exposed by a drop in surface elevation (Fig. 1). However, mineralization assays using model low-molecular-weight PAHs indicate that microorganisms present in these seep sediments are capable of degrading freshly added radiolabeled naphthalene and phenanthrene at rapid rates [4,5,7]. Despite an immense biodegradation potential at this site, PAHs anomalously persist in the sediments.

At least a portion of the naphthalene and phenanthrene in the upgradient subsurface region of this field study site is microbiologically available because metabolic adaptation has occurred. Naphthalene and phenanthrene were metabolized by microorganisms in groundwater sediments from inside but not outside the contaminated plume [4,5]. This observation, in combination with high numbers of protozoa in the groundwater contaminant plume, demonstrated in situ biodegradation of these contaminants [4]. However, substantial amounts of the naphthalene in downgradient seep sediments at this field site appear to be protected. One way of explaining the lack of naphthalene metabolism is that in the field site's setting, delivery of final electron acceptors fails to meet the catabolic demand of microbial cells. Thus, experiments contrasting aerobic and anaerobic naphthalene metabolism are warranted.

The inhibition of contaminant biodegradation by diffusion and partitioning processes in soil and sediments has been investigated for many years [10–13]. In some reports, the partitioning of organic compounds into organic matter pools in soil has been found to be caused by sorption–desorption reactions [14,15] or formation of covalent bonds as bound residues [16]. However, in many reports, the precise mechanisms of the contaminant binding has not been known. Furthermore, the relationship of bioavailability to the kinetics of these binding reactions is also not well understood. Insights into this relationship are crucial for determining the environmental fate and effects of contaminants. Recently, model contaminant compounds, phenanthrene and 4-nitrophenol, were found to be less extractable and more resistant to biodegradation as the contact time for diffusion and partitioning reactions increased [17]. This inverse relationship between the bioavailability of contaminants and the duration of partitioning reactions, termed an "aging effect," may have significant implications for explaining the persistence of biodegradable compounds generally


Upland portion of site
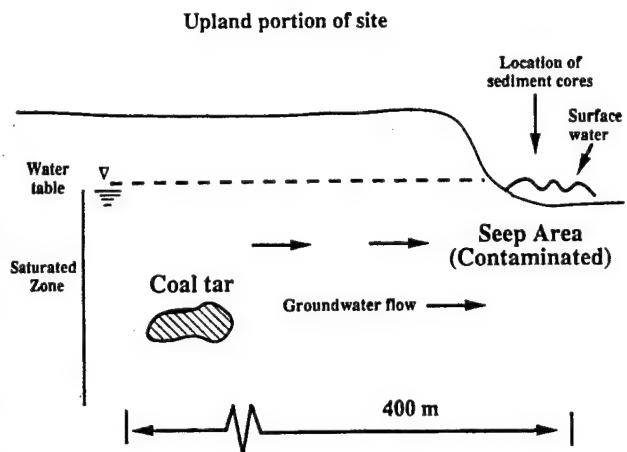
Fig. 1. Diagram of study site in vertical cross section showing source of coal tar contaminants, groundwater flow, and sampling sites. Dimensions are not to scale.

and may have specific bearing on the fate of naphthalene at our study site.

We have defined three hypotheses for explaining the persistence of the simplest of the PAH compounds, naphthalene, in sediment at our field study site: (1) the activity of the naphthalene-degrading microbial populations in the sediments is limited by either inorganic and organic nutrients or the accrual of toxic materials; (2) the native microbial populations are limited by the lack of suitable final electron acceptors; and (3) sorption and/or partitioning reactions have protected the naphthalene by diminishing its bioavailability. This study was designed to test these hypotheses using a mixture of field and laboratory approaches.

MATERIALS AND METHODS

Field site characterization

Spatial, hydrologic (Fig. 1), and chemical characteristics of the field study site have been described in detail elsewhere [4,5,18]. Vertical sediment cores were obtained by using a sledge hammer to drive two steel pipes (rigid electrical conduit pipe, 5.5 cm outer diameter 1.5 m length) through the water-saturated organic muck and sand lenses into the relatively dense underlying aquifer sand. This denser material sealed the end of the pipe, allowing recovery of intact vertical core samples. By measuring the depth of inserted pipe and the height of removed core, the degree of sediment compression was calculated and used to compute precompression sampling depths.

Accurate determination of in situ O_2 concentrations was of primary concern during coring operations. All O_2 determinations were completed in the field within 5 min of core removal using a portable O_2 probe (Microelectrodes, Inc., Londonderry, NH, USA) that was inserted either directly into surface sediments or through holes drilled into the side of the coring barrel. As the drill bit was removed, the probe was inserted into the core. Entry of atmospheric O_2 was prevented because the periphery of the hole was sealed by the slurry-like muds. Calibration of the probe was accomplished using N_2-purged water (0% O_2) and air-saturated water (21% O_2). While still at the field site, the steel pipes were segmented at selected depths using a rotary hand pipe cutter. As the segments were removed, a flamed stainless-steel spatula was used to scrape off the top layer of mud to expose undisturbed sediment beneath. A flamed stainless-steel scoop (3 cm^3 fixed volume) was used next to immediately transfer 3 cm^3 of this mud into a 20-ml precleaned glass vial (Ichem). This was followed by the addition of 2 ml butanol : hexanes (1:9) extractant, and the vial was sealed with a screw-cap Teflon®-backed septum and placed on ice. Surface water from the site was gathered using glass pipettes. Four-milliliter volumes of water were transferred to 10-ml sterile glass ampules. These were immediately amended with 1 ml of the butanol/hexanes extractant and sealed with a butane torch prior to being transported back to the laboratory. Relatively large headspace volume, low volume of added extractant, and rapid sealing of the ampules minimized the threat of an explosion. Gas chromatography analysis of naphthalene is described below. Total microscopic microorganisms were determined by fluorescence microscopy as previously described [19].

Mineralization assays

Procedures for mineralization assays have been previously described [5,7]. Under aseptic conditions, 4 to 8 g (wet weight) of contaminated seep sediment was placed in 25-ml screw-cap vials (Pierce, Rockford, IL, USA) containing a glass marble. Two milliliters of sterile deionized water and 0.05 μCi [1-^{14}C] naphthalene (10.1 mCi/mM, >98% radiopurity, from crystals allowed to dissolve in sterile deionized water; Sigma Radiochemicals, St. Louis, MO, USA) were added to the sediment. A shell vial (15 × 45 mm, Kimble, Vineland, NJ, USA) containing 0.4 ml of 0.5 N NaOH as a CO_2 trapping solution was placed on top of the marble inside the 25-ml vial, which was then sealed with a Teflon-faced silicone septum (Pierce) and a screw-cap ring. The vials were incubated in triplicate at room temperature on a rotary platform set to 30 rpm. At several time intervals after sealing the vials, the trapping solution inside was withdrawn with a needle and syringe through the septum. Trapping solution was replaced, and portions of the ^{14}C trapped were counted with a liquid scintillation counter (Model LS 5000 CE, Beckman Instruments, Inc., Fullerton, CA, USA).

Assays were designed to test the response of sediment microorganisms to a variety of nutrient amendments. The amendments included inorganic compounds (nitrate and sulfate, at a final concentration of 100 ppm each; ammonium and phosphate, 35 ppm and 5 ppm, respectively; a standard trace metal solution [20]; or a standard vitamin solution [20]) or carbon sources (nonradioactive naphthalene at 3 ppm, acetate at 30 ppm, or an aqueous dissolved organic carbon [DOC] extract of the sediment). In order to allow for the possibility that microsites in the test flasks may be O_2 limited, a treatment with headspace at twice the atmospheric level was also prepared.

Anaerobic incubations

Four grams of seep sediments, in triplicate 35-ml glass serum bottles, were amended with ^{14}C-naphthalene and 1 ml of boiled deionized water with or without 30 ppm nitrate or sulfate. These bottles were prepared using anaerobic techniques in a Coy Anaerobic Hood (Ann Arbor, MI, USA) containing a mixture of N_2:H_2 (98:2). After being sealed in the hood, the bottles were statically incubated in the dark at 24°C. One set of bottles received only water and was pressurized with pure O_2 to triple the atmospheric level. Thus, the treatment conditions were oxygen amendment (= aerobic), nonamended

(= methanogenic), sulfate amended (= sulfate reducing), and nitrate amended (= denitrifying).

Anaerobic slurry-phase naphthalene metabolism assays were prepared and implemented using high-performance liquid chromatography (HPLC) analysis as previously described [21,22]. The anaerobically prepared mineral salts, naphthalene-saturated medium' was stirred and transferred in 75-ml aliquots under anaerobic conditions to 12 120-ml serum bottles. The 12 bottles were split into four treatments (three replicates each) as follows: (1) the anaerobic medium, uninoculated with sediments from the site (the medium alone, where no activity was expected); (2) the anaerobic medium, inoculated (the "live" treatment, where activity was expected); (3) like 2 but autoclaved (to prove that the activity can be eliminated by heat and pressure); (4) like 2 but poisoned with $HgCl_2/HCl$ (to prove that the activity could be eliminated with known biotic inhibitors). High-performance liquid chromatography analyses of 1-ml fluid subsamples were used to follow loss of naphthalene from solution.

The oxygen content of headspace gases was determined by injecting 100 μl onto a Perkin Elmer model 8500 gas chromatograph equipped with a thermal conductivity detector and a molecular sieve 5A-filled column (5.5 m × 0.3 cm; Supelco). Oven temperature was constant at 40°C.

Determination of naphthalene in site sediments and waters

Analytical procedures have been previously described [7]. Vials and ampules containing extractant and site sediment and water samples were stored at 4°C until injection of 1 μl of the extractant phase into a Hewlett-Packard 5971A gas chromatograph/mass spectrometer equipped with a Nukol glass capillary column (30 m × 0.25 mm inner diameter, 0.25 μm film thickness) (Supelco, Bellefonte, PA, USA). The unit was equipped with a 5791 mass selective detector operated in scanning mode at a voltage of 2,047 V, a temperature of 300°C, and a scanning mass range of 10 to 180 *m/z*. The injector temperature was 250°C. The oven temperature began at 40°C, was held for 1 min, and then raised to 200°C at a rate of 10°C/min.

Field assay to test O_2 limitations

A randomized block design was implemented in which 40 open-ended glass tubes (11 cm height × 1.7 cm outer diameter) were inserted on 10-cm centers directly in a flat 0.5-m² area of the seep. Each cylinder was gently forced into the organic mud to a depth of 9 cm. This allowed 2 cm of the cylinder to extend above sediment surface. One-half of the cylinders were amended with 1.33 ml of a NaBr solution (12.5 g/L, used as an internal tracer to account for dilution of solutes contained by the tubes), while the other half were amended with the NaBr solution plus a freshly prepared H_2O_2 stock solution so that the final H_2O_2 concentration in the 18 ml of sediments enclosed by the tubes was 250 ppm. At sampling times, four tubes and their contents from each of the two treatments were removed from the field site, emptied into glass vials, immediately amended with 4.5 ml of hexanes/acetone extractant (1: 1), sealed with Teflon-lined caps, shaken, put on ice for transport back to the laboratory, and stored at 4°C for gas chromatography/mass spectrometry analysis of naphthalene. Bromide was measured in aqueous dilutions prepared from the sediments retrieved from the field site using a specific bromide ion electrode (Orion Instruments) using protocols and standards recommended by the manufacturer.

Table 1. Vertical profiles of naphthalene, oxygen, and microorganisms in two cores from the organic matter-rich seep area of the study site

Core	Depth (cm)	O_2[a] (mg/L)	Naphthalene (ppm)[b]	Total microbial counts (log·cm⁻³)[c]
1	0[d]	4	1.0	nd[e]
	3.5	0	2.0	9.7
	7.6	0	11	9.2
	13	0	27	9.2
	29	0	3.6	9.9
2	0[d]	4	0.9	nd[e]
	8.6	0	18	9.6
	18	0	39	9.1
	30	0	20	9.0
	51	0	45	9.4

[a] O_2 was measured by dipping the oxygen probe into on-site flowing surface water or by inserting the probe into an intact core immediately after its removal. See Materials and Methods for details.
[b] Sample extracted with 1:9 butanol:hexanes immediately after core was removed from seep. Units are micrograms of naphthalene per cubic entimeter of sediment. Naphthalene concentration for water samples represents an average of three replicate 4-ml samples gathered, extracted, and sealed in glass ampules on site. See Materials and Methods for details.
[c] Total microscopic bacterial counts performed using fluorescence microscopy (19).
[d] Surface water.
[e] nd = not determined.

Aging naphthalene with sediments

Procedures were those previously described [23,24]. Twenty-four grams of γ-sterilized (2.5 Mrad from a ⁶⁰Co source in Ward Laboratory, Cornell University) seep sediment was aseptically dispensed to 20 sterile 60-ml glass ampules. At times between 28 and 0 d prior to initiating the mineralization assays, 10⁵ dpm of aqueous ¹⁴C-naphthalene was added in 24 ml of 0.005 M $CaSO_4$ along with nonradioactive naphthalene at a final concentration of 15 ppm (one-half saturation). On day 0 the ampules were opened, the supernatant was poured off and assayed for ¹⁴C by scintillation counting, and 5-g (wet weight) quantities of the sediment were dispensed to 25-ml screw-capped glass vials for the mineralization assay described above. The inoculum was either an unenriched 1:100 dilution of seep sediment dispensed (1.0 ml) to each vial or an enrichment (1 g to 100 ml mineral salts + 30 ppm naphthalene) of cells derived from seep sediment and grown on a liquid mineral salts/naphthalene mixture for 1 d prior to being dispensed (0.5 ml) to each vial.

RESULTS

Field site conditions

The vertical profiles of oxygen, naphthalene, and microorganism concentrations in two independent cores that were removed from the seep area are shown in Table 1. It is clear from data in Table 1 that surface waters of the site were aerobic; however, only centimeters below the surface oxygen was below detection. The vertical distribution of both naphthalene and microbial cells were variable with depth and ranged from 0.9 to 45 ppm and log 9.0 to 9.9 cells/cm³, respectively.

Nutrient limitation assay under aerobic conditions

In order to test the hypothesis that naphthalene persistence in seep sediments may be caused by limiting amounts of nu-
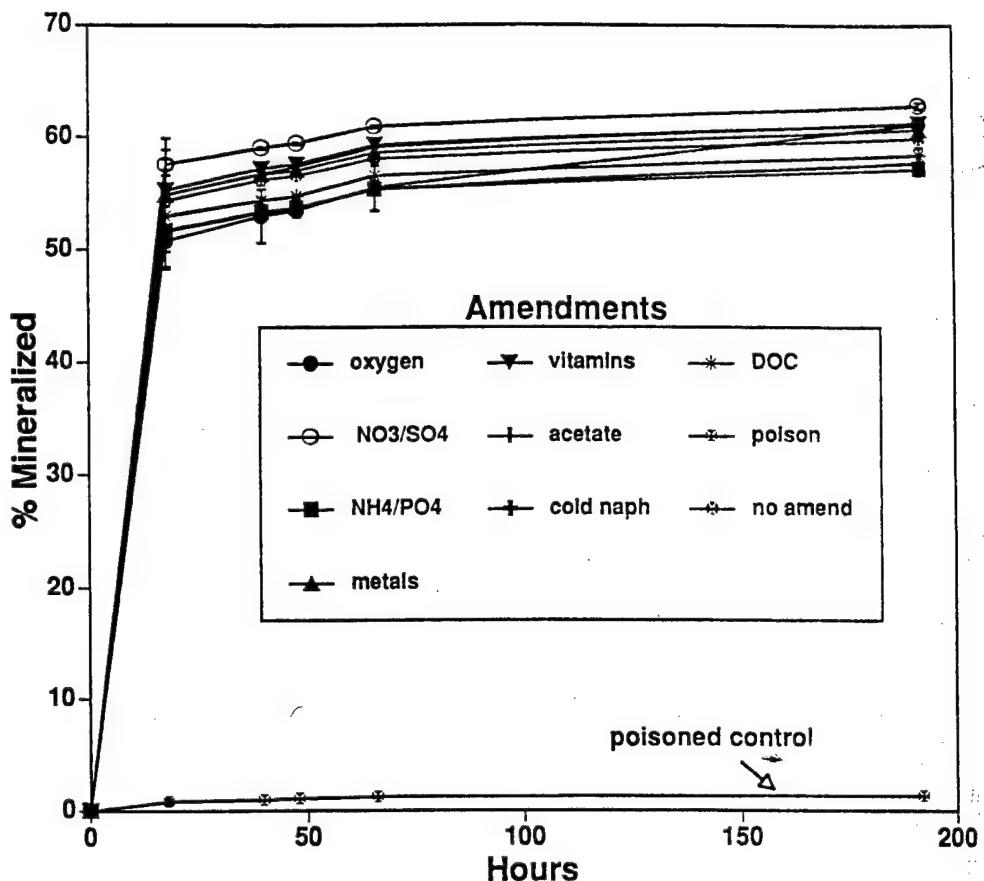
Fig. 2. Aerobic mineralization of naphthalene in seep sediments with and without amendments of a variety of potentially limiting inorganic and organic nutrients. See Materials and Methods for details.

trients and/or inhibitory substances, naphthalene mineralization assays were performed using seep sediments amended with a variety of potentially limiting nutrients. Results of the mineralization assays appear in Figure 2. No significant amounts of $^{14}CO_2$ were generated in the poisoned controls. However, all live treatments converted approx. 54% of the added radioactivity to $^{14}CO_2$ within 18 h. Relative to the unamended treatment, no significant stimulation of aerobic naphthalene mineralization was detected in treatments amended with the inorganic and organic nutrients shown in Figure 2. We conclude that microorganisms were present in the seep sediments and mineralized naphthalene at similar rates and to similar extents, regardless of the various amendments. Thus, aerobic naphthalene metabolism in laboratory-incubated site sediments does not appear to be limited by (1) the absence of naphthalene-degrading microorganisms, (2) the presence of toxic substances, (3) availability of alternative carbon sources, or (4) organic or inorganic nutrients.

Anaerobic naphthalene metabolism assays

Data in Table 1 clearly show that anaerobic conditions predominate at depth in the seep sediments. Because dioxygenase enzyme complexes (which require molecular oxygen as a cosubstrate) are essential for the known biochemical mechanisms of naphthalene metabolism [25], one obvious explanation for naphthalene's persistence at the site is simply lack of O_2. However, anaerobic metabolism of naphthalene under nitrate-reducing conditions has also been reported [26–28].

To further explore the propensity for microorganisms at our

study site to metabolize naphthalene under a variety of redox conditions, assays analogous to the aerobic naphthalene mineralization (Fig. 2) were performed under anaerobic conditions designed to favor methanogens, sulfate-reducing bacteria, and denitrifiers. An O_2-amended treatment was also prepared. Results of the 16-d incubation in which $^{14}CO_2$ production was monitored revealed that 77% of the naphthalene was converted to $^{14}CO_2$ in the bottles to which oxygen was added but not in the other treatments (data not shown). Thus, no significant naphthalene mineralization was detected in treatments designed to encourage denitrifying, sulfate-reducing, or methanogenic processes.

Because naphthalene metabolism under denitrifying conditions had been previously reported [26–28], an additional attempt using slurried sediments was implemented. The fate of naphthalene in uninoculated, autoclaved, and viable denitrifying treatments is shown in Figure 3. During the first 12 d of monitoring, the naphthalene concentration remained constant in all treatments. Thus, no evidence for denitrifying naphthalene metabolism was obtained. To prove that there were aerobic naphthalene-degrading cells present in the inoculum, on day 12 the headspace gas in two of the triplicate vials in each treatment was pressurized with O_2. Gas chromatography analyses of headspace gases confirmed that the O_2 concentration rose from <0.2% (below the detection limit) to 30%; thus, the oxygen pressurization was effective. The third vial in each triplicate was left untreated to serve as an anaerobic control. Addition of O_2 to the uninoculated, autoclaved, and poisoned treatments (data not shown for the latter) had no effect on
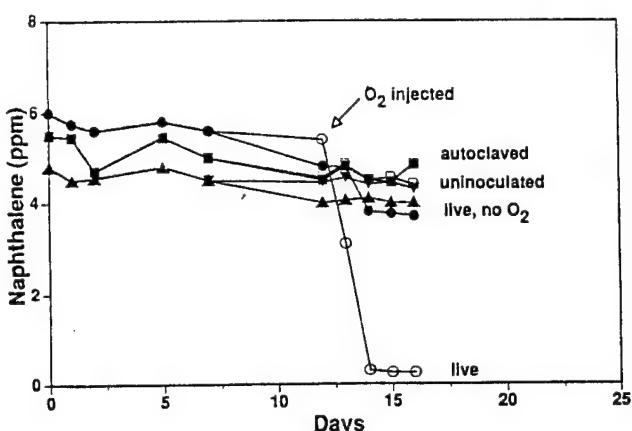
Fig. 3. Slurry-phase anaerobic incubation of aqueous naphthalene in Teflon®-sealed serum bottles. The medium contained mineral salts and KNO₃ to encourage denitrifying microorganisms. Treatments were live (inoculated), uninoculated, and autoclaved. After 12 d, the headspace in two of the three replicate bottles for each treatment was pressurized with oxygen.

naphthalene concentrations: regardless of O_2 additions, naphthalene concentrations remained constant. However, after O_2 addition to bottles containing viable microorganisms enriched for denitrification, the naphthalene concentration plummeted rapidly (Fig. 3). This loss of naphthalene did not occur in the viable cultures that did not receive O_2. Furthermore, the experiment yielding the data in Figure 3 was repeated and found to be reproducible.

Data in Figure 3 provided no evidence for denitrifying naphthalene metabolism in sediments from the seep area at the study site. Rather, data in Figure 3 unequivocally prove that O_2 was limiting naphthalene metabolism in the seep-derived microbial community. This relates directly to data in Table 1, which show that O_2 was absent under field conditions at the study site. If naphthalene metabolism under methanogenic or sulfate-reducing or denitrifying conditions was operating at the seep, each of these metabolic regimes would have responded to their respective treatments. Because only aerobic metabolism of naphthalene was observed, lack of O_2 was strongly implicated as the reason for naphthalene persistence at the study site. Thus, provision of O_2 to these sediments in field plots at the study site theoretically should have eliminated the naphthalene contamination.

Field assay to test O_2 limitations

The randomized block design field assay amended site sediments with H_2O_2. This experiment was designed to test the oxygen-limitation hypothesis by periodically removing tube-enclosed sediments from the field site and assaying the retrieved slurries for bromide and naphthalene. Results are shown in Table 2. Over the 21-d field experiment, recovered bromide concentrations diminished by a factor of 2.5, presumably due to dilution caused by both rainfall and surface water flow. Naphthalene recovered from these tube-enclosed sediments during this experiment were highly variable and roughly constant in both the H_2O_2-amended and unamended treatments (Table 2). Thus, the H_2O_2 treatment failed to enhance naphthalene disappearance in the field site.

Assessing the influence of sorption reactions on naphthalene mineralization

Results of the aging/mineralization experiment in which the inoculum was a dilution of unenriched seep microorganisms

Table 2. Naphthalene concentrations in field experiment (sediments contained by open-ended glass cylinders and amended with 250 ppm H_2O_2)

	Naphthalene in sediment (ppm)[a]	
Time (day)	$- H_2O_2$	$+ H_2O_2$
0	5.5 ± 2.6	5.2 ± 1.7
1	4.3 ± 0.9	5.9 ± 3.5
5	12.2 ± 2.4	6.2 ± 2.8
12	8.0 ± 2.0	12.9 ± 8.9
21	12.3 ± 0.2	7.6 ± 1.7

[a] Average of four replicate sediments. All concentrations of naphthalene normalized to 50 g wet weight of recovered sediment.

appear in Figure 4. The greatest rate and extent of naphthalene mineralization were found from the oldest (21- and 11-d) treatments. No simple relationship between the duration of contact time and naphthalene bioavailability occurred, as treatments aged 1, 11, and 21 d all clustered together.

Results of the aging/mineralization experiment in which the inoculum was enriched in aqueous naphthalene are shown in Figure 5. The data contrast strikingly with those in Figure 4. By changing the inoculum from unenriched to enriched, a clear relationship between rates/extents of mineralization and contact time was achieved. The greatest mineralization was observed on sediment aged 0 and 1 d. Furthermore, mineralization was generally diminished proportionately to the duration of the contact period between naphthalene and the sediment. Thus, the aging–bioavailability hypothesis seemed to apply to the sediment microbial community, after being physiologically adapted toward aqueous-phase naphthalene.

DISCUSSION

The anomalous persistence of the readily biodegradable low-molecular-weight PAH naphthalene was investigated by testing a series of hypotheses using both laboratory and field methodologies. Freshly added ¹⁴C-naphthalene was extensively converted to ¹⁴CO₂ in aerobic laboratory-flask assays regardless of a variety of organic and inorganic amendments. Thus, neither toxicity nor nutrient limitation were responsible for the absence of in situ naphthalene metabolism. Naphthalene metabolism was absent from anaerobic incubations that used both ¹⁴CO₂ trapping and HPLC analysis of slurry-phase sediments. However, O_2-amended anaerobic treatments rapidly metabo-
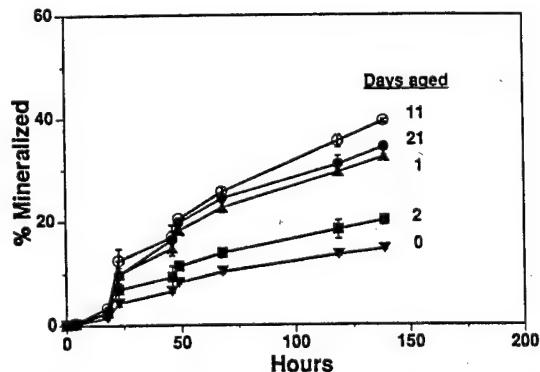

Fig. 4. Cumulative mineralization of naphthalene aseptically aged with seep sediments and subsequently inoculated with an unenriched microbial suspension from the seep. Aging times ranged from 0 to 21 d. Each data point represents the average from three replicate flasks. Error bars represent standard deviation.
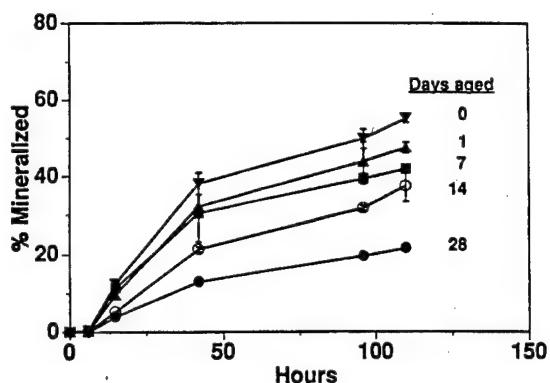
Fig. 5. Cumulative mineralization of naphthalene aseptically aged with seep sediments and subsequently inoculated with aqueous microbial enrichment suspensions from the seep. Aging times ranged from 0 to 28 d. Each data point represents the average from three replicate flasks. Error bars represent standard deviation.

lized the substrate. Therefore, O_2 limitation, as supported by O_2 probe analyses of field cores, was implicated as the explanation for field persistence of naphthalene. However, a randomized block design H_2O_2-amended field experiment failed to stimulate naphthalene loss in situ from the sediments relative to the unamended controls. Reasons for lack of stimulation of aerobic naphthalene biodegradation by the added H_2O_2 are uncertain but may include (1) spatial heterogeneity of naphthalene in the field sediments, (2) O_2 respiration at the expense of nonnaphthalene substrates in seep sediments, (3) H_2O_2 toxicity, (4) lack of O_2 limitation, (5) inaccessibility of naphthalene in the sediment to microbial attack, and (6) reactions of H_2O_2 with sediment components that may impede or bypass O_2 delivery to microorganisms [29].

This investigation failed to uncover any evidence for anaerobic naphthalene metabolism. Absence of evidence for anaerobic naphthalene metabolism is not proof that the process does not occur on site, it merely means that the procedures used were unsuccessful. Several reasons for this can be proposed: (1) the mineral salts medium either lacked key nutrients or contained inhibitory substances; (2) the organisms that carry out the process may be active only in a true three-dimensional matrix, and by attempting to measure the reaction in a dilute slurry, habitat disruption may have eliminated activity; (3) the naphthalene concentration may have been too low to support growth; (4) the denitrifying and other anaerobic naphthalene degraders were truly absent; and (5) anaerobic naphthalene metabolism may have been too slow to measure in the time frame of the experiments. It is very clear, however, that aerobic metabolism is a robust metabolic process that is likely to dominate any other naphthalene transformation whenever O_2 is available to sediment microorganisms. In fact, a unique product of dioxygenase attack on naphthalene has recently been isolated from field site waters [30].

The final approach reported here for explaining naphthalene protection from microbial metabolism was the aging–bioavailability hypothesis. This postulates that time-dependent sequestration reactions have rendered the naphthalene unavailable for microbial attack. In interpreting the data shown here (Figs. 4 and 5), it is essential to appreciate the coupled, complex physical/chemical and biological mechanisms that contribute to the net outcome of $^{14}CO_2$ production. The aging–bioavailability hypothesis is consistent with data derived from sediment inoculated with an enrichment culture from seep sed-

iment (Fig. 5). However, when an unenriched inoculum was utilized, the resultant $^{14}CO_2$ production curves showed an indifference to the duration of contact time between sorbent and substrate (Fig. 4). Reasons for these results are uncertain but probably reside in variabilities in physiological properties of mixed, unknown microbial populations. It is the microbiological component of the test system that was least understood. A major difference between the two aging experiments was how the inocula were prepared. In the first experiment (Fig. 4), microorganisms native to the seep sediment were inoculated directly into the mineralization vials before adaptation to aqueous naphthalene could be achieved, whereas in the second experiment, the inoculum was an enrichment, i.e., the microbial community was given soluble naphthalene and the opportunity to adapt to this aqueous substrate prior to inoculation. Implicit in "adaptation" is a physiological or a compositional change in the microbial community. We feel that some microorganisms may prefer, or be indifferent to, sorbed naphthalene while others may prefer it in an aqueous form. This type of physiological specialization for naphthalene metabolism has been described previously [14]. Had our experimental design employed aging periods of longer duration [15,31], perhaps the naphthalene would have been more strongly or deeply sequestered and hence less bioavailable.

Methodologies for aging of chemicals with sediments used here contrast in a variety of ways with those used by others [10–15,32,33]. The implementation of aging–bioavailability studies necessarily requires that certain logistical decisions and arbitrary choices about both materials (e.g., soils, aging vessels, aging times) and procedures (e.g., use of carrier solvents, mineralization assays, sediment extraction) be made. Differing details of methodological approaches may influence the outcome of these experiments, which are designed to explore the mechanisms by which the interactions between matrix solids and chemicals alter their bioavailability.

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EFFECTS OF SUBSTRATE MINERALOGY ON THE BIODEGRADABILITY OF FUEL COMPONENTS

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Abstract—Experiments were carried out to determine the effects of mineralogy on the biodegradability of components of a whole fuel by a soil microbial consortium. Samples of quartz sand (Fischer Sea Sand) and illite clay (API 35) were spiked with marine diesel fuel, aged, slurried, and inoculated, and concentrations of fuel components were monitored over time. To help distinguish biotic from abiotic processes, identical samples were poisoned with mercuric chloride and were run in parallel. While there was chromatographic and biomarker evidence of *n*-alkane biodegradation in the sand samples, illite samples showed no evidence of biogenic loss of aliphatic components. Polycyclic aromatic hydrocarbons, on the other hand, were lost equivalently on both minerals and in both cases were lost to a much greater extent than were total petroleum hydrocarbons (TPHs). These results suggest that *under our experimental conditions,* illite inhibited the bioavailability of some TPH components to the soil microbial consortium.

Keywords—Biodegradability   Mineralogical effects   *n*-Alkanes   Polycyclic aromatic hydrocarbons

INTRODUCTION

Petroleum-contaminated soils are highly complex systems. First, soils can be composed of naturally occurring organic matter and one or many minerals, exhibiting varying properties such as surface chemistry, grain size, and porosity. Second, petroleum products are mixtures of hundreds of aliphatic and aromatic organic compounds, the relative proportions of which vary greatly between fuel type and somewhat between batches of the same type. Each component differs in its reactivity, solubility, volatility, mineral surface affinity, and biodegradability [1–4]. Furthermore, the mode of introduction of the contaminant into the soil or sediment, postdepositional weathering, and diverse mobility characteristics of the components can drastically alter the composition of the bulk fuel contaminant. However, little work has been done to examine mineral-specific effects on bioavailability, mobility, and degradability of fuel components, which are important issues from a remediation standpoint.

Remediation technology is often developed and demonstrated on soils composed of pure, simple, and relatively inert quartz sand [5–7]. However, the bulk of natural soils are actually complex mixtures of materials, including many reactive and high-surface-area components such as clay minerals, organic matter, and metal sulfides and/or oxyhydroxides. A key factor in both the remediation and bioavailability of the most recalcitrant fractions of hazardous organic waste is the long-term sorption that can occur between organic molecules and clays or other minerals in soils and sediments [8,9]. Organic-mineral binding mechanisms are poorly understood [10], and the fundamental chemistry controlling these important issues in natural systems must be determined before it will be possible to develop an intelligent approach to remediation of contaminated sites. We have shown,

for instance, that the rate and nature of interaction and weathering [11] of fuels on pure clays and pure sands are fundamentally different, probably because of different mineral/contaminant interactions taking place in the clays and sands.

The purpose of this experiment was to take a first look at the effects of substrate mineralogy on the biodegradability of fuel components. In order to achieve this, representative "end-member" minerals, a quartz sand and an illite clay, were chosen.

MATERIALS AND METHODS

Single-component soils, Fischer Sea Sand and illite clay (API 35 [12–14]; see below for a discussion of clay organic content), were lightly ground and sieved to the same size class, 25 to 140 mesh (710–104 μm). Sand and clay were weighed in 5-g aliquots into vials, and then marine diesel fuel (DFM, Navy Fuel Depot, San Diego, CA, USA) was added to yield concentrations of 0 to ~35 mg DFM/g dry substrate. Prepared samples were then placed on a Cole-Parmer Roto-Torque for 2 weeks. The samples were used as standards for a fluorescence experiment [11] and were then stored in sealed vials, which were not airtight, for 2 years. After 2 years, individual 5-g samples were combined (all the sands together and all the clays together) and homogenized by mixing in a lapidary tumbler. Homogeneity of the combined samples was confirmed by bulk fluorescent measurements of subsamples of the materials. Concentrations after homogenization were 8.5 ± 0.2 mg/g and 11.5 ± 0.2 mg/g for sand and clay, respectively, as determined by gas chromatography with flame ionization detection (GC–FID) analysis of extracts (see method description below).

Clearly, these samples were not fully representative of field-contaminated samples since they were not subjected to environmental conditions present in natural systems. Yet, there are advantages to using spiked samples for laboratory investigations. Because extractions of well-aged, field-contaminated samples are never quantitative, it is impossible to be certain what was in the samples to start with. With spiked samples, accurate

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contamination levels are known, so mass balance considerations are simpler. Thus, the process of spiking samples to mimic natural samples represents a compromise, some level of control (and knowledge of what was in the system to start) in exchange for less "reality" in the samples.

Because these samples were designed for fluorescence rather than biodegradation experiments, no attempt was made to keep the samples abiotic. However, the availability of well-aged, homogenized monomineralic materials spiked with a well-characterized fuel and the observed poor biodegradability of total petroleum hydrocarbons (TPHs) in clay-rich soils [15] led us to carry out biodegradation experiments on these materials.

In order to carry out biodegradability experiments, aliquots of these aged, homogenized samples were then slurried. Slurries were prepared by adding one part soil (1 g) to two parts basal salts solution (2 ml) in 20-ml sterile vials with foil lids. Sufficient samples were prepared to sacrifice triplicate samples at designated times over the course of the 99-d experiment. The slurries were inoculated with a population of DFM-acclimated soil microorganisms. The microorganisms were originally obtained from a jet fuel-contaminated site and subsequently grown on DFM as the sole organic carbon source.

Selecting for well-acclimated DFM degraders was important for our experiment. Typically, when given a mixture of petroleum hydrocarbons, bacterial populations will begin to degrade alkanes before they attack polycyclic aromatic hydrocarbons (PAHs). When it appeared that the microorganisms were utilizing the PAHs in the DFM broth, the population was transferred to fresh DFM medium. The indicator for PAH degradation was the appearance of an orange color in the bacterial broth. An orange color commonly appears in growth medium when there is an accumulation of 1-hydroxy-2-naphthoic acid, a phenanthrene degradation product [16]. To enhance the selection process for well-acclimated DFM degraders, the organisms were allowed to grow on the fresh DFM until an orange color appeared again, at which time the bacterial population was used as the inoculum for the experiment.

Abiotic controls were prepared by adding mercuric chloride to the slurries [17]. Sterility of the controls was confirmed by lack of microbial growth on tryptic soy agar (TSA) plates. It is important to note that all approaches for generating sterile controls in biodegradation experiments have limitations. The addition of poisons, autoclaving, and gamma irradiation all can change the biogeochemistry of the system in some way [10]. Thus, biotic and abiotic samples cannot be deemed to be absolutely comparable. However, if results are carefully interpreted, abiotic controls can prove to be valuable.

The slurry vials were shaken at 250 rpm on a reciprocal shaker under ambient laboratory conditions. Triplicate samples were periodically sacrificed for cell density determination and chemical analyses. Microbial densities were based on the colony forming units (CFUs) of serial dilutions on TSA plates.

To prepare samples for TPH and PAH analyses, slurries were acidified with 100 μl of concentrated hydrochloric acid and then extracted for 3 min with 16 ml of a 1:1 mixture of methanol and hexane. The hexane extracts were then spiked with *tert*-butylbenzene as an internal standard and analyzed by GC–FID for TPH content and by GC–mass spectrometry (MS) for PAH analysis.

Both forms of GC analyses were carried out on Hewlett Packard 5890 gas chromatographs equipped with 12-m × 0.2-mm inner diameter capillary columns with 0.33 μm thickness of cross-linked methyl silicone gum. For GC–FID analysis the temperature program started at 35°C for 0.6 min and was ramped 10°C/min to 180°C and then 20°C/min to 230°C. The temperature was held at 230°C for a total run time of 30 min.

For GC–MS analysis the starting temperature was held at 35°C for 0.6 min and was ramped 20°C/min to 240°C, where it was held for a total run time of 15 min. The MS detector was run in the selective ion mode with chosen m/z values correlating with the parent ion of those PAHs and other unsaturated cyclic compounds known to be in DFM. Dimethyldibenzothiophene, a component of DFM that is fairly resistant to biodegradation, was treated as a conserved internal biomarker or "built-in" internal standard.

To be used as a conserved tracer, the compound must be resistant to the weathering process and thus must be relatively stable toward microbial degradation. Large molecules of low volatility and low solubility are good candidates. Compounds in this category that are constituents of DFM and have been suggested for this use are methylated phenanthrenes and dibenzothiophenes [18]. We opted to use dimethyldibenzothiophene as a conserved tracer and internal standard due to our numerous observations of its persistence in laboratory degradation studies and also because of evidence of its persistence under more severe conditions in the field over periods longer than the course of this study [19].

Polycyclic aromatic hydrocarbon data are reported as the area of the GC–MS peak normalized to the area of dimethyldibenzothiophene. Substituted aromatics are reported as summed values for all similar structures. For example, all the methylnaphthalenes are summed and reported as C-1-naphthalenes; C-2-naphthalenes are the sum of dimethyl- and ethylnaphthalenes, and so on. When data are reported as zero, they represent nondetectable levels of the analyte. Error bars are the standard deviations about the means. In all cases where error bars are not visible on graphs, they are smaller than the data symbols.

Mineral characterization

The clay samples were not individual mineral grains at the size class used in this experiment but were clumps or aggregates of grains which adhered together as the result of minor, possibly calcareous, cementation. The sands, however, consisted of individual mineral grains, primarily of quartz (>99.9%). Figure 1 shows scanning electron microscope photographs of both the illite and Fischer Sea Sand at the same magnification. Measurements of the specific surface areas of sand and illite at the mesh sizes used were carried out by adsorption of N_2 gas (20–22) and showed that illite has a significantly higher surface area (~78 m²/g) than quartz sand (~0.2 m²/g) [11].

Although we find no discussion in the literature [12–14], there is evidence that the API 35 illite may contain some natural organic matter. Carbon-hydrogen-nitrogen (CHN) measurements on the illite showed 1.6 to 3.5% C by weight and 0.13 to 0.28% N by weight. Carbon dioxide coulometry reveals that the illite has 0.0 to 0.3% by weight inorganic (carbonate) carbon, suggesting, by difference, that there is up to 1.3 to 3.5% organic carbon by weight in the illite samples. In previous related work, fluorescence peaks were sometimes observed in illite-fuel extracts that were not observed in sand-fuel extracts, suggesting some, possibly organic, fluorophores in the illite. However, we have not been able to reproduce those peaks in extracts of illite without fuel, possibly because the presence of the fuel enhanced the extraction of these natural organic components. It is probable that some natural organic matter occurs in the illite, which may either provide a carbon source for some of the microbes or may

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mask clay effects. No C or N were observed in CHN measurements of the sand samples.

Ideally, experiments should have been carried out with the organic matter removed from the illite to decouple effects of the clay and the organic matter. However, attempts to remove well-aged natural organic matter from porous materials without significantly changing the mineral character are largely ineffective [23,24]. In our experience, attempts to remove organic matter by use of a muffle furnace (at 500°C, which should remove organic matter without changing the clay mineral structure) resulted in an illite clay which would strongly acidify slurries, killing microbial populations. This is probably the result of hydroxyl groups being driven off the mineral surface at high temperature (as confirmed by infrared spectroscopy, J. Kubicki, personal communication). Thus, this and subsequent experiments were carried out with the organic matter still in the illite.

RESULTS AND DISCUSSION

Experimental limitations

It should be pointed out that one potential difficulty of using aged, contaminated materials is reduced recovery of analytes due to weathering and time. It is well known that due to sorption processes, analyte recovery decreases with the aging of samples [11,25–27]. This can make mass balance considerations especially difficult. Furthermore, since the production of biosurfactants during slurry treatment may increase extractability over time [28], this may further confound interpretation of biodegradation experiments. Thus, it is important to remember that as an experiment progresses extractability may change and that all concentrations reported are extractable concentrations and not necessarily total concentrations.

In this experiment, biodegradability of fuel components in a whole fuel is determined by changes in analyte concentrations in extracts over time, relative to poisoned controls. We emphasize that a lower value in the biotic relative to the abiotic extracts does not prove biodegradation since, as stated above, simply adding a poison may change the slurry characteristics relative to the nonpoisoned sample, and uncontrolled or unanticipated losses may be driving observed changes. It does, however, provide a strong suggestion of biodegradation. Only the detection of degradation products or the mineralization of labeled compounds would be definitive evidence of biodegradation. However, in whole fuel mixtures, this can be quite difficult. In a mixture such as DFM, because of the complexity of the chromatograms (see Fig. 2), degradation products are difficult to track. The other option, labeling, allows for the tracking of only one component at a time, so a massive number of experiments would have to be mounted to track a variety of individual components in a whole fuel mixture. Furthermore, there is a growing body of literature on the limitations of unaged, spiked, or labeled materials as analogues for field-contaminated systems [10,29].

All of the factors described above should be borne in mind when the results of this and other experiments are interpreted. However, because "real" field systems are complex and because we will never encounter situations in the field that reflect the neat, laboratory systems we can completely control and quantify, we choose to trade some level of certainty for insight into complex systems.

As stated above, the spiked sand and illite samples were allowed to weather in vials for about 2 years before the experiment was begun. Thus, day 0 results as reported in the following figures are actually from well-aged materials. Since the vials were not airtight and the samples were not sterile, some volatilization, and perhaps degradation, of the DFM components on the substrates occurred even before this experiment began. Different degrees of volatile loss from the sand and illite are apparent in the GC–FID chromatograms in Figure 2. The "Clay–Initial" sample shows less evidence of volatilization, with taller and more abundant peaks on the left-hand, lighter end of the chromatogram, when compared to the "Sand–Initial" chromatogram. This suggests that the clays, or the oils retained on the clays, better retain the volatile components, inhibiting volatile loss. This also means that the sand and clay samples, though always treated the same, started the biodegradation experiment with different compositions (e.g., the clay had a higher TPH concentration and a larger proportion of volatile and biologically labile components). This will again be apparent in later sections when figures of TPH and PAHs are compared in sands and clays.

Microbiological results

Figure 3 shows microbial densities in log CFUs/ml. Note that degraders of specific hydrocarbon types (e.g., alkanes or PAHs) were neither counted nor identified but rather that densities were counted to monitor the general microbial health of the system. Day 0 reflects the microbial densities in the slurries just after being inoculated with the DFM-acclimated organisms. Within a day of inoculation, populations on both substrates rose to near 10^8 CFUs/ml. Bacteria levels remained vigorous throughout the experiment, dropping slightly near the end, possibly reflecting a depletion of mineral salts and/or production of toxic by-products. Of note is the fact that microbial populations were as high (or higher) in the clay samples than in the sand samples. The slightly higher bacterial counts may be the result of the higher surface areas presented by the clays, the higher TPH levels in the clay samples throughout the experiment, or both.

Prior to adding the inoculum, background levels of microorganisms for the sand and clay were <10 and <1,000 CFUs/ml, respectively, suggesting that the materials had very low microbial populations before inoculation. The colony morphologies of the background organisms were distinct from the DFM-acclimated inoculum. From the time of inoculation and onward, the unique morphologies of the background colonies were not apparent in the spread plates. Thus, the inoculum apparently dominated the population throughout the course of the experiment.

TPH results

The bottom portion of Figure 2 shows the chromatograms of the sand and clay extracts after 99 d in bioslurries. The chromatograms of the sand and clay extracts differ in important ways. First, the sand has significantly fewer and smaller peaks standing above the unresolved complex mixture (UCM), or hump of the chromatogram. These depleted peaks represent the *n*-alkanes, which generally disappear rapidly when biodegradation occurs. This suggests that the normal alkane portion of DFM on sand was biodegraded over the course of the experiment. The clay sample, on the other hand, still contained high levels of *n*-alkanes, even after 99 d in the slurry. Though some of the lighter peaks on the left side of the chromatogram have decreased and total concentration has reduced, the chromatogram seems to reflect volatile loss more than a classic pattern of biodegradation. In fact, the clay chromatogram after 99 d looks much like the sand chromatogram did at day 0.

Total petroleum hydrocarbon concentrations in the poisoned
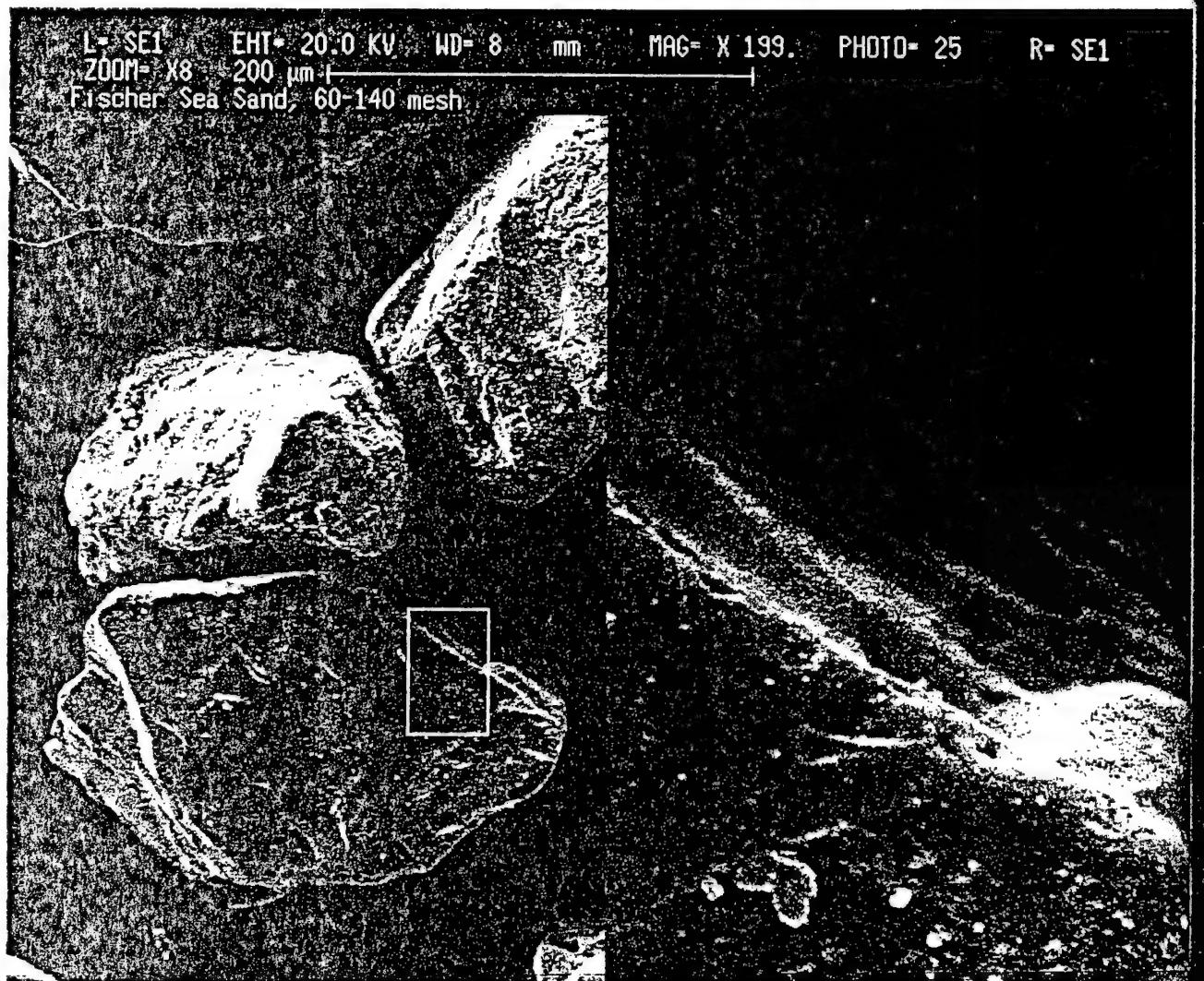
Fig. 1. Scanning electron microscope images of Fischer Sea Sand (**above**) and API 35 illite (**facing page**). Images are magnified to 200×, and images to the right are 8× magnifications of the framed portions.

and biotic samples are plotted in Figure 4. Poisoned controls were measured only for days 0 and 99. Lines connecting the data points are for visual purposes only and are not meant to provide any mechanistic insight into projected trends between these days. It is not likely that these trends were actually linear. Percent extractable TPH over time drops in both the sands and clays by about 30 to 40%. When compared to their respective poisoned controls, the biotic samples appear to have lost only about 10% additional TPH, not a very significant offset. Only visual examination of the chromatograms in Figure 2 makes a compelling argument for differences between the sand and illite samples. The TPH drop in the poisoned controls reflects primarily volatile loss, as was reflected in the chromatograms (not shown here).

· The apparent lack of biodegradation of the TPH components on the illite when compared to the sand is further bolstered by n-C-18 to phytane ratios from the chromatograms over time. These ratios have traditionally been used to distinguish biodegradation from abiogenic processes such as physical weathering, volatilization, and leaching [30–32]. The straight chain alkane (C-18) is readily biodegradable and has a volatility that is similar to its mated branched alkane, phytane, which can be

less biodegradable. That phytane is relatively resistant to biodegradation in this system is evidenced by the fact that it comprises one of the only remaining peaks visible above the UCM for the highly degraded "Sand–99 day" chromatogram in Figure 2. A drop in the C-18/phytane ratio indicates biodegradative loss of the n-alkane. Figure 5 shows that the C-18/phytane ratio for the illite samples and both poisoned controls stay flat for the 99 d of the experiment. Statistics show no significant drop from day 0 to 99 for these samples (paired t-test, one tail, $\alpha = 0.05$); thus, there is no evidence of biodegradative loss of C-18. However, the sand samples drop rapidly from about 2.5 to about 0.3 in 8 d, approaching the detection limit for C-18 above the UCM, suggesting rapid and extensive degradation of the n-alkane. The C-18/phytane ratio in conjunction with the chromatograms strongly suggest that TPH biodegradation is either not occurring or is occurring at very low rates in the illite samples.

Examination of Figures 2, 4, and 5 suggests that, based on the classic GC methods for and indicators of fuel biodegradation, the API 35 illite strongly inhibits bioremediation of fuel components and that any loss observed appears to be a volatile loss. Since, as discussed above, even in small proportions clay
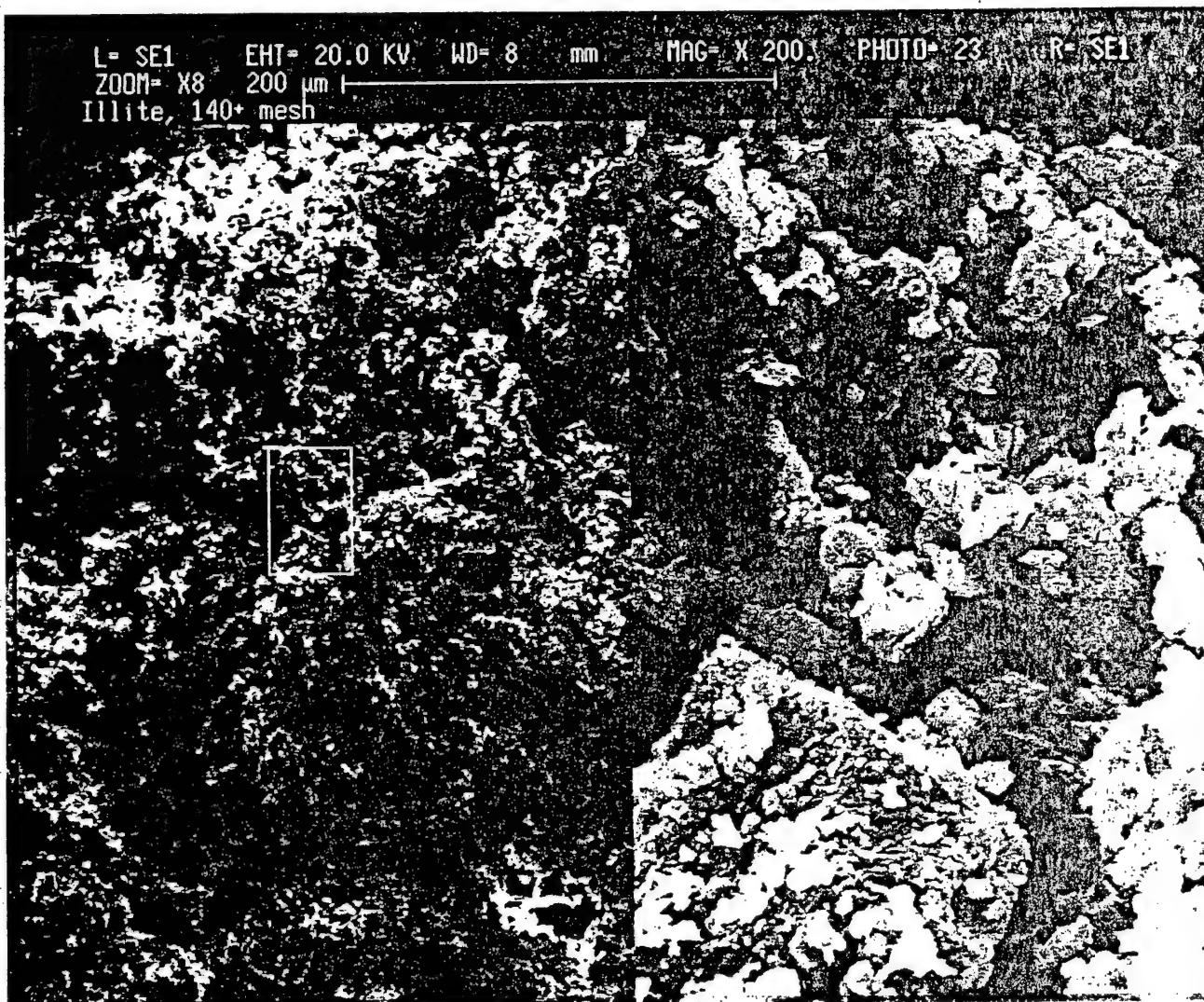
Fig. 1. Continued.

in a soil or sediment can dominate the surface area, and possibly contaminant behavior, this clay effect could potentially dominate alkane biodegradability in an illite-containing soil or sediment. In other words, the clay could make alkanes less bioavailable. However, the microbiological results reflected comparable organism densities in the clay and the sand samples, suggesting the utilization of some form of organic matter in the clay samples.

PAH results

Because PAHs comprise a potentially toxic and carcinogenic fraction of many petroleum fuels [33], they have merited much attention in recent years. It has been demonstrated that soils and sediments exposed to fuel contamination often contain PAH-degrading organisms [34] and that under favorable conditions PAH degradation can occur at a measurable rate [35,36]. The more toxic, multiring compounds, however, are often subject to slow or negligible degradation rates due to their chemical complexity [35,36] and tendency to partition to solid substrates [10,37].

Fresh DFM is composed of about 6% PAHs by weight. Since PAHs are a minor component of the fuel, trends in their behavior tend to be buried in the UCM of the GC–FID chromatograms.

Figures 6 through 9 show the concentrations of various PAHs in extracts over the course of the experiment based on GC–MS analyses. All PAHs that were detected and measurable in the samples are reported. The simplest and most volatile aromatics in DFM, such as naphthalene, were not observed in these samples, probably because they had ample time to volatilize during sample weathering. Those PAHs which were observed will be discussed from the simplest to the most complex.

C-1-naphthalenes are among the most volatile PAHs detected in the samples. This volatility is apparent from the nearly complete loss of this component in the sand samples during sample weathering, as seen in the much lower concentrations in the sand than the clay samples at day 0 (Fig. 6a). On both substrates concentrations rapidly drop to very low levels in the first 10 to 20 d. It is not possible, however, to determine whether this loss was by biodegradation or volatilization, since the poisoned controls dropped to similarly low levels by day 99. It should be pointed out that having analyte concentrations in abiotic and biotic samples dropping to the same low level does not necessarily indicate that biodegradation is not occurring in the live samples but rather that the combination of degradation and volatilization in the biotic samples is not occurring at extents greater than the volatilization alone in the killed samples.
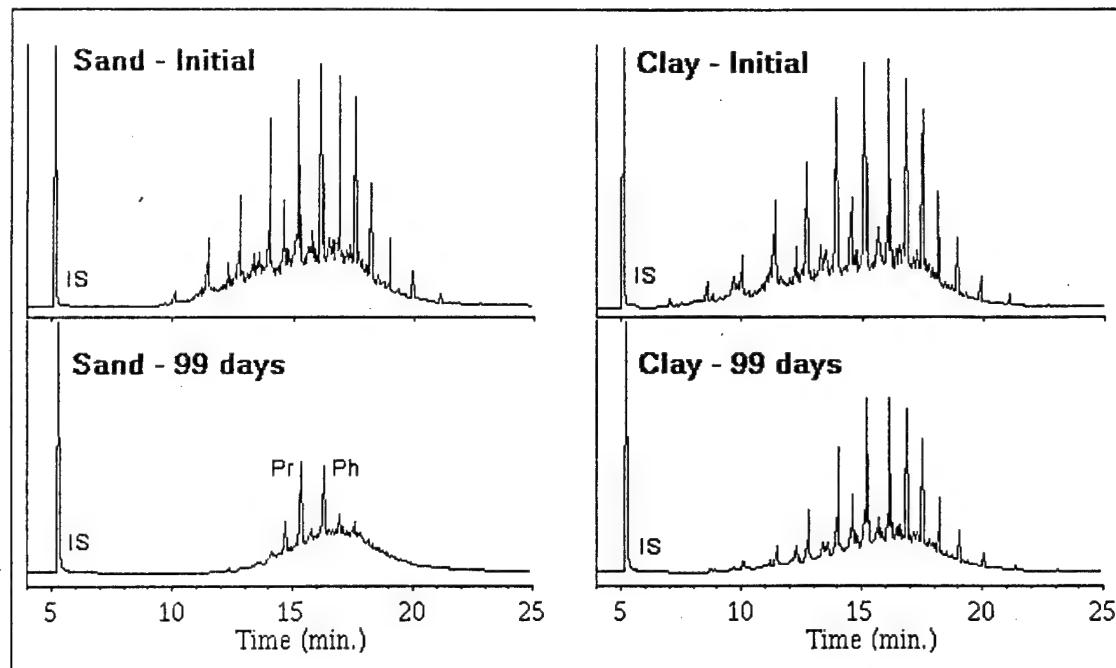
Fig. 2. Gas chromatography with flame ionization detection chromatograms of sand and clay extracts at days 0 and 99. Labeled peaks are the internal standard (IS), pristane (Pr), and phytane (Ph).

C-2-naphthalenes (Fig. 6b) are somewhat less volatile than C-1-naphthalenes. Loss of these components is very rapid on both substrates, dropping to near the detection limit in the first 5 d. Although the sand poisoned controls drop to as low a level as the live sand samples, the clay poisoned controls do not drop as low. This suggests two things, that the illite inhibits complete volatilization of the C-2-naphthalenes (reflected in the clay control not dropping to below detection as did the sand control) and that some biodegradation of this PAH may be occurring on the live illite samples (reflected by the live samples having lower values than the killed samples). C-3-naphthalenes tell a similar story (Fig. 6c), but the slight offset between the live and killed sand samples suggests that some biodegradation, in addition to volatilization, is occurring on the sand. However, it should again be noted that since the $t = 0$ levels of the C-2-naphthalenes were much higher in clays than in sands, they cannot be directly compared.

C-1-fluorenes (Fig. 7) drop off precipitously in the first 4 to 5 d in both the sand and clay samples. However, as for C-2-

naphthalenes, the abiotic and biotic sand samples at day 99 are indistinguishable. In the clay samples, however, the offset between killed and live samples is substantial, with killed samples showing very little reduction and live samples dropping below the detection limit. Just as for C-2-naphthalenes, this suggests both that the clays can inhibit volatilization and that extensive biodegradation may be occurring in the live samples.

Anthracene and phenanthrene are indistinguishable by our current GC–MS method, as are their C-1, C-2, and C-3 structures, so they are reported together. For both anthracene/phenanthrene and C-1-anthracenes/C-1-phenanthrenes (Figs 8a and b), the sand and clay live samples drop below the detection limit in about 20 d or more at similar rates. However, both sand and clay killed samples show only negligible drops over the 99 d
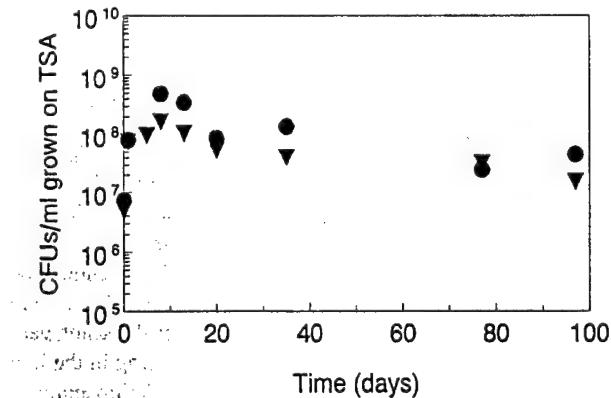
Fig. 3. Microbial densities versus days in slurry. Filled circles are inoculated clays, and filled triangles are inoculated sands.
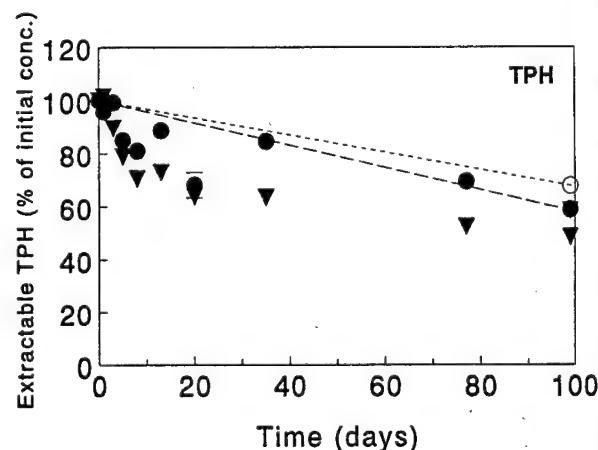
Fig. 4. Extractable total petroleum hydrocarbon concentrations as a percentage of initial concentrations versus days in slurry. Filled circles are inoculated clays, filled triangles are inoculated sands, and open symbols are the corresponding poisoned controls. In this and the following figures, error bars that are not visible are smaller than the symbols.
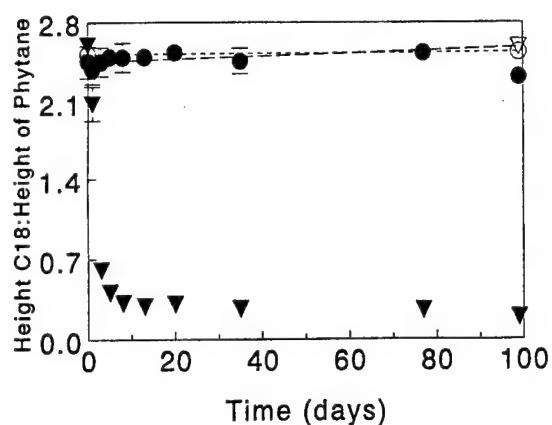
Fig. 5. C-18/phytane ratios in extracts versus days in slurry. Filled circles are inoculated clays, filled triangles are inoculated sands, and open symbols are the corresponding poisoned controls.

of the experiment. These significant offsets between the live and killed samples strongly suggest that biodegradation of these compounds is occurring on both substrates.

C-2-anthracenes and C-2-phenanthrenes (Fig. 8c, again reported together) show very small drops to similar levels on both substrates in the live samples. In both abiotic sets of controls, the drops are similarly small. These compounds are somewhat resistant to biodegradation; thus, in neither of the substrates does the level drop very much. The slightly greater offset between the live and poisoned clay samples is probably due to differences in initial oil concentrations. C-3-anthracenes and C-3-phenanthrenes (Fig. 8d, reported together) are at levels near the detection limit of our method, so no clear statement can be made about whether these PAHs are decreasing or staying constant for any of the samples.

The sum of the measured PAHs provides further insight into the PAH behavior on these substrates. Live samples of both sand and clay (Fig. 9a) drop to about the same level, possibly suggesting that this is a level at which the organisms in this mixture cease to respond to the PAHs or that conditions in the sample vials are no longer conducive to microbial activity. Killed illite samples, which started at a higher level and with a higher proportion of volatile components than did the sand samples, show a slightly greater relative drop than do the killed sand samples, but both result in total PAH (TPAH) that is clearly above that in the live samples. When plotted as percent of initial extractable TPAH, live sand samples drop by about 80%, while clay samples drop about 90% (Fig. 9b). It should be noted again that clays started out with a higher concentration of volatile and biologically labile components, which may explain some of this offset since they both level off at the same final concentrations (Fig. 9a). The percent lost in both poisoned controls is only about 30%, suggesting that biodegradation may account for at least 50 to 60% of the TPAH loss in the live sand and clay samples, all other things being equal.

There are two separate but related issues which need to be addressed to explain biodegradation patterns in these experiments. The first is the different degree of degradation of the TPH relative to that of PAH components. The second is the difference in TPH behavior in the sand and clay. The observed differences of the alkane and aromatic behavior on sands and clays can be explained in several ways. These include differential water solubilities, surface affinities, or molecular sizes (and thus the ability to "hide" in mineral pores). Aromatics are
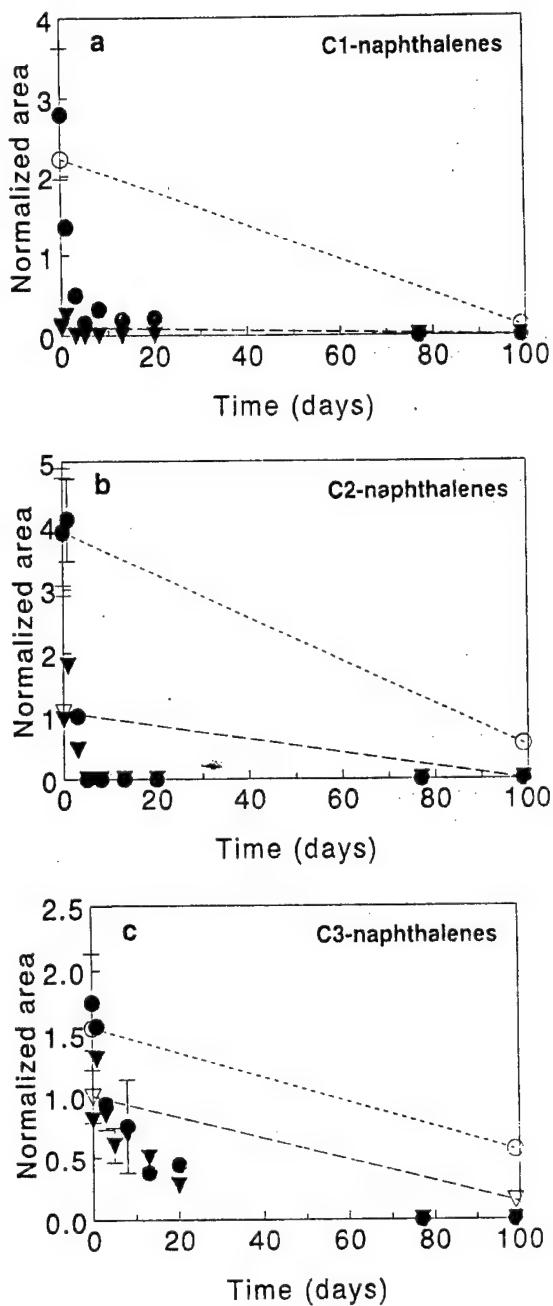
Fig. 6. (a) C-1-naphthalenes, (b) C-2-naphthalenes, and (c) C-3-naphthalenes in extracts versus days in slurry. Filled circles are inoculated clays, filled triangles are inoculated sands, and open symbols are the corresponding poisoned controls.

more water soluble than alkanes of similar molecular weight and may be less subject to partitioning onto the clay surfaces and thus may be more bioavailable. Solubility (or leachibility) may to an extent control the relative degradability of the PAHs, since the extent of degradability seems well correlated with the solubility of the various alkyl-substituted PAHs, which is correlated to leachibility. On the other hand, it can be argued that the relative biodegradability of the PAHs is driven by structural controls as well [29].

It is unclear in this case whether the biodegradability of the *n*-alkanes on the illite is driven by controls such as sorption, occlusion, relative solubility, steric hindrances, or feeding preferences. Most likely it is a complex interaction of these, but
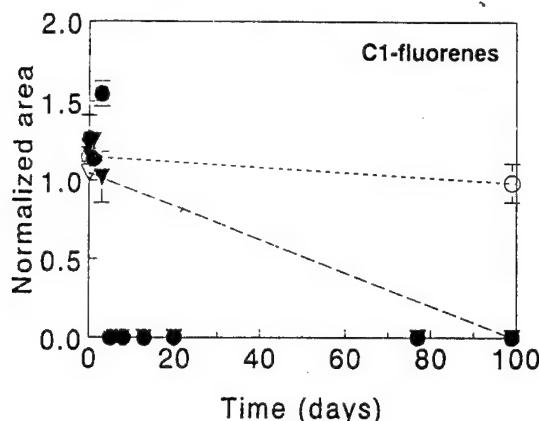
Fig. 7. C-1-fluorenes in extracts versus days in slurry. Filled circles are inoculated clays, filled triangles are inoculated sands, and open symbols are the corresponding poisoned controls.

most workers focus on one aspect of these interrelated issues at a time. A detailed picture of bonding between organic molecules and minerals remains elusive [38]. Pinpointing the chemistry in soils and sediments is particularly difficult because both the organic component (humins, humic acids, fulvic acids, and any contaminant present) and the fine-grained minerals and colloids are generally poorly characterized [10]. It is also possible that the resistance to degradation of the TPH components relative to the PAHs is a result of the microbial consortium which was applied. Although the consortium was acclimated to whole DFM, the community may be better adapted to degrade PAHs than TPH. In related experiments (Apitz et al., unpublished data), a San Diego Bay sediment consortium biodegraded TPH components in DFM as effectively as it degraded PAH components.

Total mineral surface areas within a soil may be extremely large, especially in clay-rich soils, due to small grain sizes and irregular surfaces with a high degree of microporosity [11,39]. Due to the large differences in sand and clay specific surface areas (e.g., illite's specific surface area is 400× greater than sand's), fine-grained minerals, even if in small proportions, can comprise the bulk of the surface area of soils. For instance, less than 1% illite by weight in sand will double the specific surface area of the resultant mixture. When soils and sediments are separated by grain size, the bulk of contamination is generally concentrated on the fine-grained clay particles [8,40–42]. Thus, even in primarily coarse materials, contaminant behavior may be controlled by the fines, principally the clays and humic materials. These components can form either physical or chemical interactions with contaminant components which may strongly affect contaminant bioavailability, transport, extractability, and degradability [10,43–49]. The rate of release of these contaminants into the environment, and thus the relative risk in each case, may differ as a function of mineralogy. In some cases, the concentration of organic contaminants that may remain tightly bound to these surfaces may exceed cleanup levels or inhibit cleanup rates so that remediation is prohibitively expensive.

Some models for the adsorption of organic species onto clays and other minerals assume that naturally present humic and fulvic acids coat the mineral surfaces [40,50–52], and then contaminants interact with that organic coating. A common organic acid, acetic acid, becomes strongly bound to some mineral surfaces and forms soluble organo–metallic complexes with the

minerals [53,54], but the rate of organic-clay reactions is relatively slow compared to the timescales of most experiment Sites that have been contaminated for years or decades have had ample time to develop intimately entwined chains of p troleum hydrocarbons and clay minerals or recalcitrant organ acids. Long-term kinetics must be addressed in experiments order to adequately understand processes in the natural env ronment. For this reason, artificially spiked samples which a intended to model reality should be allowed to age for lo periods of time [25,26].

Two common models for the sorption of organic molecul onto minerals, which may affect contaminant bioavailabili are the equilibrium partitioning model and the two-site, no equilibrium sorption model. The equilibrium partitioning mod is straightforward and can explain most sorption of organi species in soils and sediments [50,55,56], but it fails for tw important components in natural systems, organic acids [2] a high molecular weight organics (e.g., polychlorinated biphe yls) [57]. Complete desorption of organics can take days, a the longer the organics have been interacting with the sedimen the longer the recovery period [58]. Consequently, kinetics sorption reactions may be affecting bioavailability and thus d gradability.

Other kinetic models for the recalcitrant portion of the sorb organics are often based on diffusion-limited transport. For e ample, Wu and Gschwend [59] and Ball and Roberts [60] su gest that diffusion of organic molecules into the solid phase responsible for slow desorption rates. Alternatively, diffusio through organic gels or colloids and out of micropores in fin grained sediments is cited as a rate-limiting step in extractin the recalcitrant fraction [38]. It is not clear how diffusion large organic molecules into solids occurs or why diffusio through gels and micropores should be so slow (1×10^{-14} cm²/ [61]. However, Johnston and Vala [62] have recently demo strated chemisorption of aromatic compounds on Cu-montm rillonite similar to the strong alkoxide binding that takes plac in zeolites and other catalysts [63,64], so chemisorption mech anisms may be able to explain the existence of slow or inhibit degradation.

In addition to sorption onto the clay surfaces, another mod which has been invoked to explain the decreased availabili (whether by extraction, biodegradation, or oxidation) of som organics in the presence of clays is physical occlusion. In e periments to remove organic matter in soils by high-energ ultraviolet photooxidation, Skjemstad et al. [23] observed th 20 to 40% could not be photooxidized. They proposed that th proportion was shielded by mineral microaggregates. Sturz [2 observed that attempts to use low-temperature oxygen plasm to remove organic carbon from fine-grained Guaymas Bas sediments failed to remove more than about half of the organ matter, possibly for the same reasons. She hypothesized th fractions of the sediment organic matter were more labile th others.

Another possible physical mechanism that may occlude o ganics is sorption into intraparticle pore spaces, interlayers, etc large enough for some organics molecules but too small f subsequent extraction by either solvents or microbes. This somewhat akin to minerals such as zeolites behaving as m lecular sieves, removing straight-chain alkanes from other p troleum components, including branched alkanes [65 and re erences therein]. It is not clear whether clays behave in a mann analogous to zeolites. For example, Ischenko et al. [66] report removal of *n*-paraffins by zeolites made from kaolinite but n

Fig. 8. (a) Anthracene and phenanthrene, (b) C-1-anthracenes and C-1-phenanthrenes, (c) C-2-anthracenes and C-2-phenanthrenes, and (d) C-3-anthracenes and C-3-phenanthrenes in extracts versus days in slurry. Filled circles are inoculated clays, filled triangles are inoculated sands, and open symbols are the corresponding poisoned controls.
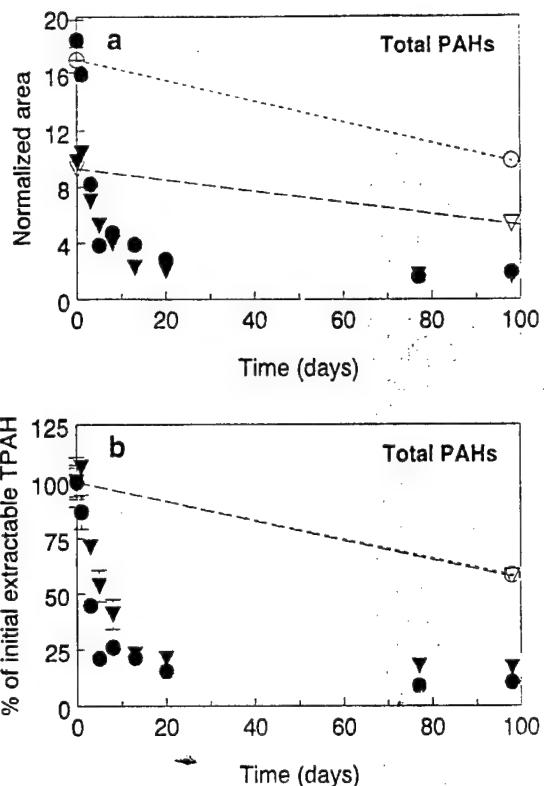
Fig. 9. Sum of all polycyclic aromatic hydrocarbons (PAHs) measured (a) as the sum of peak areas normalized to dimethyldibenzothiophene and (b) as a function of percent of initial extractable total PAH versus days in slurry. Filled circles are inoculated clays, filled triangles are inoculated sands, and open symbols are the corresponding poisoned controls.

by kaolinite itself. However, some clays, such as smectites, have expandable interlayers that allow absorption of organics, and others have enough surface roughness that organic molecules may fit into pits on the surface. Hence, the physical occlusion mechanism in clays merits investigation.

All these physicochemical processes may affect bioavailability of some organics, but different microbial species may have novel approaches for dealing with these barriers. Thus, even if the soil consortium in this system was unable to utilize *n*-alkanes on illite, another consortium may have been unaffected.

In a thorough survey of soil–microbial interactions, Stotzky [44] noted 10 years ago that the understanding of clay–organic–microbial interactions is not very extensive and quite equivocal. When organics are sorbed to clays, either reduced or enhanced bioavailability can result, depending on mineralogy, organic composition, and experimental conditions. Sometimes clays can act as concentrators and thus sources of nutrients, while at other times they can inhibit mineralization. What controls these processes remains unclear and practically unexamined for petroleum components. Stotzky states that ". . . the paucity of data on the adsorption and binding of hydrophobic substrates on clay minerals and on the subsequent microbial utilization of these substrates indicates that more studies with hydrophobic compounds and clay minerals—both 'clean' and 'dirty'—are warranted." In spite of an intervening decade since this was written, it still holds true. It is clear that an understanding of mineral-organic interactions may be critical to the success or failure of

bioremediation attempts in soils and sediments, so they merit further study.

CONCLUSIONS

By classic analysis, there appeared to be minimal biodegradation of TPH and alkanes on illite. In spite of these dire observations, there is strong evidence that PAHs degrade equally well on the illite and the sand samples. Based on percent loss from original concentrations, PAHs show much more extensive loss (80–90%) than TPH (30–40%) on both substrates. Since PAHs are often the contaminants of greater concern because of potential toxic effects and regulatory scrutiny, this is an exciting result. It is also an indication that simple measures of degradation may not tell the complete story in complex systems.

The above results show clear offsets in the biodegradative potential of DFM components on sand and illite in this particular system, but several issues remain to be resolved. Illite represents only one of many clay mineralogies. It was chosen for our experiments because it is the most abundant clay in San Diego area soils and sediments [67], which are being used in several experiments here, but other clays, such as montmorillonites and kaolinites, need to be examined, as well as metal sulfides and oxyhydroxides. Labeled compounds need to be used to unequivocally demonstrate biodegradation and to help perform complete mass balances. The role of natural organic matter versus mineralogy needs to be clarified, and whole soils and sediments must be examined more carefully. Furthermore, a different microbial consortium may not respond to the mineral effects in the same way.

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BIOAVAILABILITY OF HYDROPHOBIC ORGANIC COMPOUNDS FROM NONAQUEOUS-PHASE LIQUIDS: THE BIODEGRADATION OF NAPHTHALENE FROM COAL TAR

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Abstract—The literature on microbial degradation of polycyclic aromatic hydrocarbon (PAH) compounds in soils and sediments shows that microbial biotreatment rates are highly variable. Although it is believed that these disparate results are somehow attributable to the bioavailability of PAH compounds, current understanding does not allow prediction of this phenomenon. This work evaluated relationships between coal tar composition and aqueous naphthalene concentration and between the rate of naphthalene mass transfer and microbial mineralization of naphthalene. The use of a dissolution–degradation framework for identifying rate-controlling phenomena in slurry biotreatment of coal tar is discussed. Experiments were performed in slurry systems with two coal tars for which naphthalene was the principal component. In slurry systems coal tar was present either as a single 0.7-ml globule or as coated onto 250-μm-diameter microporous silica beads. Independent tests were conducted with these systems to assess the rates of naphthalene mass transfer and rates of naphthalene biomineralization. The area-dependent mass transfer coefficient of naphthalene in the coal tar–silica beads system was three orders of magnitude greater than for the single coal tar globule. A coupled, solute–mass transfer biodegradation model was used to compare initial biomineralization rates with rates of naphthalene dissolution. Analysis of the results showed that the rate of biomineralization of naphthalene in the systems containing single coal tar globules was influenced by the rate of mass transfer of naphthalene.

Keywords—Biodegradation   Mass transfer   Naphthalene   Coal tar   Nonaqueous-phase liquid   Bioavailability

INTRODUCTION

Coal tar is a by-product of coal coking and gasification processes and is often associated with subsurface contamination at many former manufactured gas plant (MGP) sites [1]. Coal tar is a multicomponent, nonaqueous-phase liquid (NAPL) composed of aromatic compounds, including various polycyclic aromatic hydrocarbons (PAHs). Coal tar may be immobilized in the subsurface as entrapped pools, as ganglia in soil macropores, or as a residual saturation in soil micropores. The immobilized coal tar may serve as a long-term source of soil and groundwater contamination due to the slow dissolution of PAH compounds and other solutes. This report discusses a framework for identifying the nature of the rate-limiting processes governing biodegradation of naphthalene from coal tar globules and from a coal tar-coated microporous matrix. Physicochemical and biokinetic rates are assessed by measuring rates of dissolution and biomineralization in mixed batch systems.

BIOAVAILABILITY OF PAH COMPOUNDS

The consensus from various investigations with PAH compounds and soils is that two- through five-ring PAH compounds can be biodegraded in liquid cultures given suitable physicochemical conditions and environments that sustain appropriate microbial populations [2,3]. However, as explained below, current understanding is very incomplete regarding achievable treatment rates and end points for microbial treatment of PAH mixtures in contaminated soil. Various laboratory tests describe PAH degradation in well-controlled systems, but how this translates into understanding what may happen in field tests is unclear. This is a particular concern with "real-world," aged samples comprising mixtures of PAHs that may exhibit much slower release of PAHs in comparison to tests with freshly applied material. The slower release of PAHs may result from a combination of physical and chemical factors controlling solubilization, desorption, and diffusion. Consequently, the design of soil treatment systems for PAH compounds requires site-specific field tests. Laboratory tests with site-contaminated samples in aerobic slurry systems may provide an indication of maximum potential rates and feasible biotreatment end points.

Microbial degradation of PAH from coal tar

Studies on biodegradation of PAHs from contaminated MGP site soils [4,5] report unpredictable and incomplete degradation of the PAHs associated with soil, although PAHs present in aqueous solutions may degrade readily. Such studies have hypothesized that mass transfer limitations may result from slow solubilization of PAHs from residual weathered NAPLs or from sorption-retarded diffusion from micropores and solid surfaces to the aqueous phase, which together may cause the PAHs to be unavailable to microorganisms in the aqueous phase.

Nakles et al. [6] reported results from laboratory biodegradation studies with contaminated MGP site soils in pan (unmixed) and slurry (mixed) systems. In soils containing 25% fines less than 75 μm, the slurry system showed a larger extent and a faster rate of degradation of total PAHs than unmixed systems. It was believed that the slurry reactor helped to promote disaggregation of larger aggregates while providing limitations to nutrient or oxygen mass transfer. In another so

containing entrapped NAPL, pan and slurry reactors approached the same overall extent of PAH removal, although the rate of degradation in the slurry reactor was greater than in the pan systems. It was concluded that mixing enhanced dissolution of PAHs from the NAPL and that the residual PAH was probably trapped inside the NAPL and would be solubilized very slowly.

Other studies on PAH degradation from MGP site soils have described the aqueous-soluble fraction of PAHs as being "readily available" and degradable with the residual PAHs as being a "resistant fraction" [7]. In those studies additional tests were performed to verify whether the lack of degradation of the residual fraction was due to mass transfer limitations or toxicity by spiking some contaminated soil with phenanthrene. The tests showed that the freshly added phenanthrene was bioavailable and degraded readily. It was concluded that the microbial population was incapable of degrading the resistant PAH fraction in the contaminated soil. This type of investigation points to mass transfer limitations as inhibiting the bioavailability of PAH compounds and thereby preventing degradation of PAHs from contaminated MGP site soils.

Erickson et al. [8] observed no significant depletion of PAHs from contaminated MGP site soil samples, including tar-contaminated soils. Supplements with additional substrates or inoculum had no significant effect on the results obtained from batch slurry tests. Additional tests were carried out with different amounts of PAH-contaminated MGP site soils being mixed with a clean soil having a microbial population capable of degrading PAHs, and, as in the work of Morgan et al. [7], some of these soil mixtures were spiked with naphthalene and phenanthrene. Naphthalene and phenanthrene equal to the amount freshly added were eliminated, while the concentrations of PAH equal to those originally present in the contaminated soil were not. This observation led to the conclusion that the contaminated soils were not toxic to the microorganisms. Polycyclic aromatic hydrocarbons from the contaminated soil were not extractable by water, and this suggested PAHs were bound to the soil in a way that the compounds were unavailable to the microorganisms. This is an extreme case in which poor bioavailability completely prevented microbial degradation.

The above studies have indicated qualitatively that mass transfer limitations may prevent significant biodegradation of PAHs from MGP site contaminated soils. Moreover, biodegradation of complex chemical mixtures such as coal tar may be further complicated by substrate interactions causing unpredictable biodegradation patterns. For example, Alvarez and Vogel [9] have reported both inhibited and enhanced biodegradation of individual BTX compounds (benzene, toluene, and xylene) in the presence of other BTX compounds. The degradation of benzene was enhanced by the presence of toluene but was inhibited in the presence of *p*-xylene.

Biotreatment of PAHs in extended field trials

Although data are limited, the biotreatment of PAH in field trials may show a "hockey stick" effect on a plot of PAH concentrations versus time. Total PAH, i.e., as represented mainly by two-, three-, and four-ring PAH, may decrease in overall concentration relatively rapidly over several months and then level off at a residual plateau concentration. It is unknown how this plateau concentration may change with time. In one fairly well-documented study, PAH-contaminated soil was treated in a field test plot from May 1986 through December 1987 at a former creosote wood preserving site [10]. During the period of active biotreatment, 1986–1987, total PAH was reduced from

an initial range of 1,200 to 3,500 mg/kg to about 800 to 1,200 mg/kg, with an average reduction of about 50%. During the 6-year period from 1987 to 1993, the treated soil was left in place and unattended. Sampling in 1993 showed that total PAH had reduced from about 1,000 mg/kg to about 200 mg/kg, along with some reductions in the five- and six-ring PAH concentrations, during the 6-year unattended period. These data illustrate that gradual reductions in PAH may continue at a very slow rate over a number of years in land treatment systems.

Scope of this research

In work described here, independent measurements of the rates of dissolution and biodegradation have been made to provide a quantitative comparison of the rate-limiting process governing biodegradation of PAH solutes from a coal tar–NAPL phase. For these particular tests, the interpretation is applicable to slurry systems in which the rate of mass transfer owing to intraparticle, sorption-retarded diffusion is small compared to the rate of external mass transfer. The study evaluates the relationships between the physical processes of equilibrium partitioning of solutes and mass transfer of solutes between the NAPL and aqueous phases and the effect on these partitioning processes on the rates of biodegradation.

Coal tar globules and coal tar-coated microporous silica particles were used in the experiments. A schematic of the systems with coal tar-coated microporous silica particles and with a coal tar globule is shown in Figure 1. In these systems, naphthalene and other solutes dissolve into the bulk aqueous phase from the coal tar phase. As a working hypothesis it is assumed that primarily naphthalene in the aqueous phase is available to the microorganisms. The bulk-phase naphthalene is degraded by bacteria, which are size-excluded from the silica micropores. In the case of coal tar globules, the bacteria are assumed to be reside either on the surface of the NAPL or in the aqueous phase.

CONCEPTUAL MODEL DEVELOPMENT FOR SLURRY BIOTREATMENT

Several factors may influence the overall rate of biotransformation of PAH compounds released from coal tar. These factors include physicochemical phenomena related to dissolution and mass transfer of PAH compounds from the organic phase to the bulk aqueous phase and biokinetic phenomena pertaining to the intrinsic microbial degradation rates. A framework for identifying the rate-controlling phenomena for the biodegradation of a PAH compound is presented in Figure 2. This figure explains how a pairwise comparison of sequentially occurring rate processes may be used to identify the rate-limiting process governing biodegradation. The figure shows a comparison of the ratio of the three dimensionless rate parameters, the Biot number, the Thiele number, and the Damkohler number. The Biot number compares external mass transfer to maximum intraparticle diffusive rate, which if greater than unity directs attention to the ratio of the biokinetic rate to the diffusive rate (the Thiele number) or which if less than unity directs attention to the ratio of the biokinetic rate constant to the rate of external mass transfer (the Damkohler number). Depending on the value of the Thiele number or the Damkohler number, the rate of the biodegradation may be governed by physicochemical or biokinetic phenomena. Knowledge of the ratio of these transport processes allows the development of simplified two-step or one-step models.

In the experiments described in this report, the biodegradation

a) Coal tar-coated microporous silica

Pore sorption
and diffusion

PAH dissolution at
NAPL interface

NAPL phase

Bulk phase transfer

PAH degradation

Bacterium

b) Coal tar globule

Coal Tar
Globule

PAH dissolution at
NAPL interface

Bulk phase transfer

PAH degradation

Bacterium
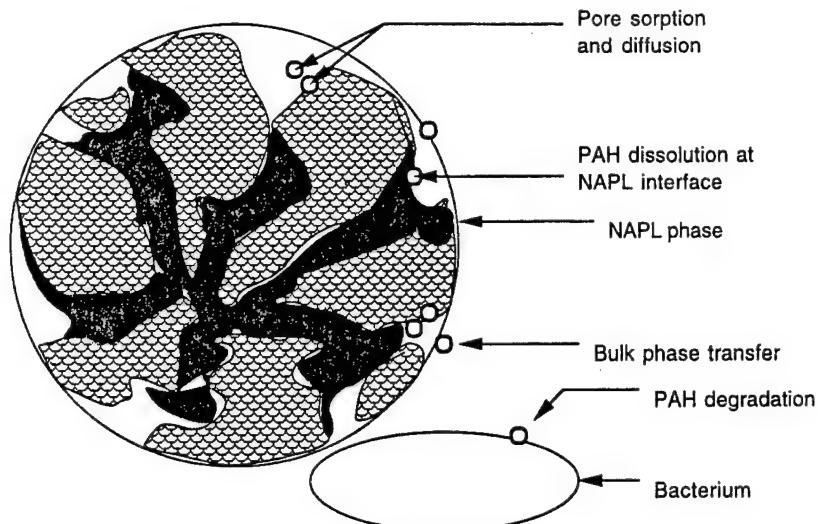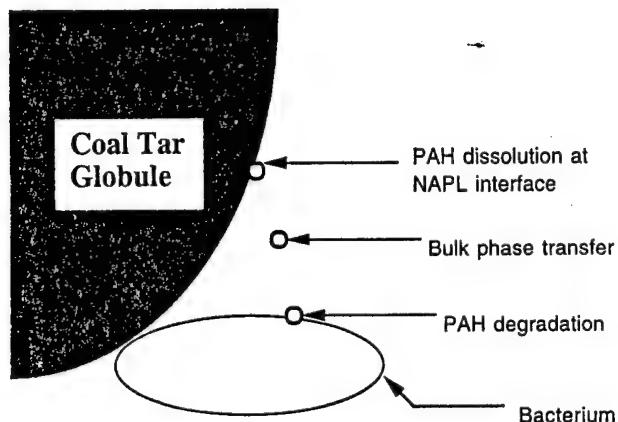
Fig. 1. Schematic showing systems studied in the experiments. (a) Coal tar-coated microporous silica beads. (b) Coal tar globule (adapted from Ramaswami [11]).

of naphthalene from coal tar was studied in slurry systems comprising tar globules or small-diameter, coal tar-coated microporous silica. Computational analysis indicated that the Biot number was less than unity, ranging from less than 0.01 to approx. 0.5, which called attention to the value of the Damkohler number for purposes of comparing the biokinetic rate to the

max flux at particle surface
max diffusive rate

>1   <1

max biokinetic rate
max diffusive rate

max biokinetic rate
max flux at particle surface

>1   <1   >1

pore sorption /
diffusion control

biokinetic
control

bulk phase
transport control

Fig. 2. Framework for identifying rate-controlling phenomena (adapted from Ramaswami [11]).

bulk external mass transfer rate [11]. Thus, a two-step dissolution–degradation model was developed using a lumped mass transfer rate coefficient to describe external surface mass transfer and a pseudo–first-order biokinetic rate constant to describe biodegradation.

The dynamic changes in aqueous phase PAH concentration $C_{(t)}$ (M/L^3), is described as

$$\frac{dC_{(t)}}{dt} = k_1 a[C_{eq(t)} - C_{(t)}] - k_{bio}C_{(t)}$$

where the first term on the right-hand side represents the rate of input of PAH to water due to dissolution from coal tar, and the second term represents the rate of removal of bulk aqueous phase PAH due to biodegradation. The biodegradation rate is expressed as a pseudo–first-order biokinetic coefficient, k_{bio} (T^{-1}). The dissolution rate is represented by a lumped mass transfer rate coefficient, $k_1 a$ (T^{-1}) that incorporates the specific surface area for mass transfer, a (L^2/L^3), and a linear driving force term that represents the departure of the aqueous concen-

tration, $C_{(t)}$, from the equilibrium concentration, $C_{eq(t)}$. The aqueous-phase concentration of a PAH compound in equilibrium with the NAPL is predicted by Raoult's law as

$$C_{eq(t)} = X_{(t)} \, C^{PAH}_{pure\ liquid} \qquad (2)$$

where $C_{eq(t)}$ is the equilibrium aqueous-phase concentration of an NAPL-derived PAH compound, $C_{eq(t)}$ is the mole fraction of the PAH compound in the NAPL at time t, and $C^{PAH}_{pure\ liquid}$ is the solubility of the pure subcooled liquid compound in water. For the coal tars used in this study, there was comparable agreement between naphthalene solubility predicted by Raoult's law and the measured equilibrium aqueous concentrations. If a significant amount of the NAPL is essentially insoluble, as with coal tars, the PAH mole fraction in the NAPL and the equilibrium aqueous-phase PAH concentration decreases as cumulatively increasing quantities of the PAH compound are solubilized and degraded by microbes. The equilibrium concentration at any time, $C_{eq(t)}$, may be calculated from Equation 2 if the fraction of depleted PAH is known.

The overall rate of biotransformation of a PAH compound like naphthalene is controlled by the slower of either mass transfer or degradation processes. It can be shown that when $k_{bio} \ll k_1 a$, i.e., mass transfer occurs much faster than biodegradation, the aqueous-phase concentration of naphthalene in the reactor is close to equilibrium such that $C_{(t)} \approx C_{eq(t)}$. Assuming negligible growth of microorganisms, the initial rate of degradation of naphthalene is given as

$$\frac{dC_{(t)}}{dt} = k_{bio}C_{(t)} \approx k_{bio}C_{eq(t)} \quad \text{for} \quad k_{bio} \ll k_1 a \qquad (3)$$

Conversely, when $k_1 a \ll k_{bio}$, i.e., when mass transfer occurs much slower than biodegradation, analysis of Equation 1 shows that the aqueous-phase naphthalene concentration in the reactor is small compared to the equilibrium concentration, and the initial rate of degradation of naphthalene is given by

$$\frac{dC_{(t)}}{dt} = k_1 a C_{eq(t)} \quad \text{for} \quad k_1 a \ll k_{bio} \qquad (4)$$

Equation 4 represents a system in which the overall biotransformation rate is controlled by mass transfer processes. For such a condition, the slope of a curve showing the extent of biomineralization versus time is directly related to the mass transfer rate coefficient, $k_1 a$.

The ratio of the mass transfer rate coefficient to the biokinetic coefficient is the Damkohler number, Da, which can be used to identify whether mass transfer phenomena or biological processes pose a limiting constraint on biotransformation rates. This dimensionless ratio can be expressed as

$$Da = \frac{k_{bio}}{k_1 a} \qquad (5)$$

which indicates mass transfer control for values much greater than unity and biokinetic control for values much less than unity. The value of the Damkohler number may be computed from estimates of mass transfer and biodegradation rate constants as discussed below. Values of intrinsic biodegradation rates for different PAH compounds may also be obtained from literature. For example, estimates of naphthalene degradation rate constant range from approx. 1/d [12] to 25/d [13].

Estimation of mass transfer rate coefficients

Mass transfer coefficients for transport from the surface of spherical bodies to the bulk aqueous phase may be estimated from several correlations available in the chemical engineering literature [14]. The lumped mass transfer coefficient for a PAH compound released from the surface of coal tar-imbibed particles, or globules of coal tar, in a gently mixed aqueous medium may be approximated by [11]

$$k_1 a = k_1 \times a \approx k_1 n_p 4\pi r^2 \approx n_p 4\pi r D_{m,aq} + \text{mixing effects} \qquad (6)$$

where k_1 is the area-specific mass transfer coefficient; a is the surface area per unit volume for mass transfer, which is related to the spherical surface area, $4\pi r^2$, of the particle or globule in contact with water, and the number density of particles or globules per unit volume of water, n_p; $D_{m,aq}$ is the diffusion coefficient of the PAH compound in water. In Equation 6, the limiting value of k_1 is taken to be $D_{m,aq}/r$ for mass transport from spheres in minimally mixed systems with no impedance for release of solute from the NAPL phase. The above correlation gives a theoretical estimate of the mass transfer rate coefficient in mixed slurry systems. This estimate was compared with measured mass transfer rate coefficients for naphthalene dissolution from coal tar-coated microporous silica beads and single 0.7-ml coal tar globules.

Estimation of biokinetic rate coefficients

An apparent value of the first-order biokinetic rate constant, k_{bio}, may be determined by fitting the dissolution–degradation model to the data obtained from biodegradation experiments. In this case, the dissolution–degradation model provides approximate values of maximum biodegradation rates for comparison with measured mass transfer rates. These values are then used to assess whether naphthalene mineralization in each system was limited initially by mass transfer or biokinetic phenomena.

In the biodegradation experiments discussed later in this report, the percent mineralization of naphthalene was measured, and equations were developed to describe the mineralization of naphthalene. The rate of mass fraction of naphthalene mineralized, $dP_{(t)}/dt$, is given by

$$\frac{dP_{(t)}}{dt} = \frac{F_m k_{bio} C_{(t)} V}{M_0} \qquad (7)$$

where F_m is the mass of naphthalene mineralized per unit mass of naphthalene degraded, M_0 is the mass of naphthalene present in the NAPL at time $t = 0$, and $k_{bio}C_{(t)}V$ is the mass of naphthalene degraded in time Δt. The magnitude of F_m was determined from the mass balance analysis for naphthalene for the coal tar globule systems at the end of the mineralization tests [15,16]. The value of F_m was 0.83 for the Stroudsburg tar and 0.89 for the Baltimore tar. A mineralization curve may be generated by numerically solving Equations 1, 2, and 7 for $P_{(t)}$ and expressing $P_{(t)}$ as a percent. The mass of naphthalene in the coal tar is known from chemical analysis of the coal tar, and since the tests were initiated with the naphthalene in the aqueous phase being in equilibrium with the NAPL, the initial conditions [M_0, C_0, X_0, and $C_{eq(0)}$] were known. The value of $k_1 a$ was determined a priori from independent experiments. The only unknown parameter, the biokinetic rate constant k_{bio}, was fitted to the mineralization data using a computer program for a modified Levenberg-Marquardt method of nonlinear least squares regression [17]. Only data for the initial part of the mineralization curves were used to obtain estimates of k_{bio}. Based on observations that about 12% naphthalene was lost due to sample handling and volatilization during the active mineralization period, the rate of naphthalene depletion was adjusted for such losses [16]. The

Damkohler number, Da, for each system was calculated from estimated values of k_{bio} and measured values of $k_l a$ to assess whether mass transfer or biokinetics controlled the overall rate of biotransformation.

EXPERIMENTAL METHODS

The coal tars used in these experiments were obtained from former manufactured gas plant sites located in Stroudsburg, Pennsylvania, and Baltimore, Maryland, USA. The coal tars were free flowing, denser than water, and primarily a mixture of PAHs with naphthalene as the most abundant PAH compound (2.2% w/w for the Stroudsburg coal tar and 10% w/w for the Baltimore coal tar) as determined by gas chromatography–mass spectrometry analysis [16,18]. Radiolabeled ^{14}C-1-naphthalene (Sigma Chemical Co., St. Louis, MO, USA) was added to the coal tars and imbibed into microporous silica beads. The microporous silica beads (PQ Corp., Valley Forge, PA, USA, mean diameter ~250 μm, average pore diameter = 140 Å), was used as a model microporous medium. Mass transfer and biodegradation experiments were conducted using radiolabeled techniques with both coal tar-containing microporous media as well as globules of coal tar.

Batch tests were conducted to measure the initial aqueous-phase solubility of naphthalene from coal tar. An initial equilibrium aqueous-phase naphthalene solubility of 3.8 mg/L for the Stroudsburg tar and 18.5 mg/L for the Baltimore tar was obtained, which agreed with the theoretical predictions from Raoult's law. The experimental data on dissolution kinetics was obtained using gently stirred flow-through reactors at different residence times [11,19]. These reactors were operated over short intervals that resulted in negligible changes in the effluent naphthalene concentrations, and thus quasi–steady-state conditions were assumed for estimation of the mass transfer coefficient. $C_{eq(t)}$ was assumed to be constant as a very small fraction of naphthalene was depleted from the coal tar during the time interval over which data for estimation of the mass transfer coefficient were collected.

Biomineralization experiments were performed under aerobic conditions in 250-ml biometer flasks. In slurry tests, silica beads were imbided with coal tar spiked with radiolabeled ^{14}C-1-naphthalene and added to each biometer with 50 ml of nutrient medium. Tests with coal tar globules employed a single globule of 0.7 ml coal tar and 50 ml of nutrient media. Biometers were inoculated with an actively growing bacterial culture (RET PA101) containing approx. 10^8 naphthalene-degrading organisms. RET PA101 is a mixed bacterial culture that has been used in other studies [16,20,21]. Radiolabeled CO_2 from biomineralization of naphthalene was trapped in the NaOH contained in the side arm of the biometers. The NaOH was sampled periodically, and its activity was measured with a Beckman LS 5000TD scintillation counter. Mineralization profiles were obtained by plotting the fraction of ^{14}C-naphthalene mineralized with time. The pathways for biodegradation of naphthalene presented in the literature involve complete ring fission to readily degradable intermediates [2,22]. Thus, it was assumed that rates and extent of mineralization of the ^{14}C-1 was representative of all carbon atoms in naphthalene.

RESULTS AND DISCUSSION

For the system with coal tar imbibed into microporous silica, a lumped naphthalene mass transfer coefficient, $k_l a$, from the flow-through tests was 4,550/d for the Baltimore tar and 3,550/d for the Stroudsburg tar. For the systems where the coal tar was present as a 0.7-ml coal tar globule, the mass transfer rate coefficient in flow-through tests was 2.0/d for the Stroudsburg coal tar and 1.6/d for the Baltimore tar. These measurements are in the range of values predicted by Equation 6. An overall value of $k_l a$ of approx. 10,000/d was predicted for the coal tar coated microporous silica beads ($n_p = 1.4 \times 10^4$ per ml). For the single coal tar globule of volume 0.7 ml, an average value of $k_l a$ of 2.4/d was predicted for the two tars [11]. Mixing conditions in the coal tar globule and coal tar–silica beads systems were very similar, and the difference in the mass transfer coefficients for naphthalene is primarily due to the difference in the coal tar–water interfacial area. The difference in the total interfacial area is reflected in difference in the magnitudes of the parameters n_p and r in Equation 6.

Typical naphthalene mineralization profiles obtained from the experiments with the coal tar-coated silica beads are shown in Figure 3a. Rapid mineralization of naphthalene in the first d was followed by an extended period of slow mineralization. Results for naphthalene mineralization profiles from experiments with single 0.7-ml coal tar globules are shown in Figure 3b. These profiles are significantly different from those obtained with the coal tar-coated silica beads. Mineralization occurred at a slower, constant rate over approx. 120 d. The mineralization of naphthalene was measured over a period of 150 d, and approx. 70% of the naphthalene in the coal tar globules was mineralized over this period. Variations in the mineralization patterns from duplicate biometers were less than 5%.

At the point where biomineralization activity became negligible, approx. 70% of the naphthalene was mineralized, and only 6 to 7% of the initial mass of naphthalene remained in the coal tar globule. This indicates substantial depletion of the naphthalene from the coal tar phase and suggests that under ideal conditions significant fractions of naphthalene can be biodegraded from coal tar NAPL. Naphthalene mineralization experiments performed with naphthalene dissolved in an insoluble NAPL, 2,2,4,4,6,8,8-heptamethylnonane, also showed that naphthalene could be essentially entirely depleted from the NAPL matrix [15]. Recovery of ^{14}C from the coal tar system ranged from 82 to 94% with an average of 84%.

The rate-controlling phenomena for the different NAPL–water systems were identified by comparing the magnitudes of the mass transfer rate coefficient, $k_l a$, and the apparent biokinetic rate constant, k_{bio}. The value of k_{bio} estimated from the initial part of the mineralization profiles shown in Figure 3a for the systems with coal tar and microporous silica beads were 14.7/d and 26.1/d for the Baltimore tar and the Stroudsburg tar, respectively. The magnitude of these biokinetic rate constants are much smaller than the mass transfer rate coefficients of approx. 4,000/d measured in the flow-through tests. The resulting Damkohler numbers are 0.004 and 0.007 for the Baltimore and Stroudsburg tars, respectively, and being much less than unity provide evidence that the initial rate of naphthalene biotransformation in such tests was controlled by the intrinsic biokinetics of the microorganisms. The first-order biokinetic rate constants obtained from the mineralization data are in the range of first-order biokinetic constants of 1 to 25/d reported for some slurry systems [12,13].

For the coal tar globule, the rates of mass transfer were slower than the biokinetic rates. The measured values of $k_l a$ were 1.6 and 2/d, which were less than the estimated k_{bio} values of 15 to 26/d obtained with the coal tar-imbibed silica beads. The resulting Damkohler numbers for these systems are 9 and 13 for the Baltimore and Stroudsburg tars, respectively, and being
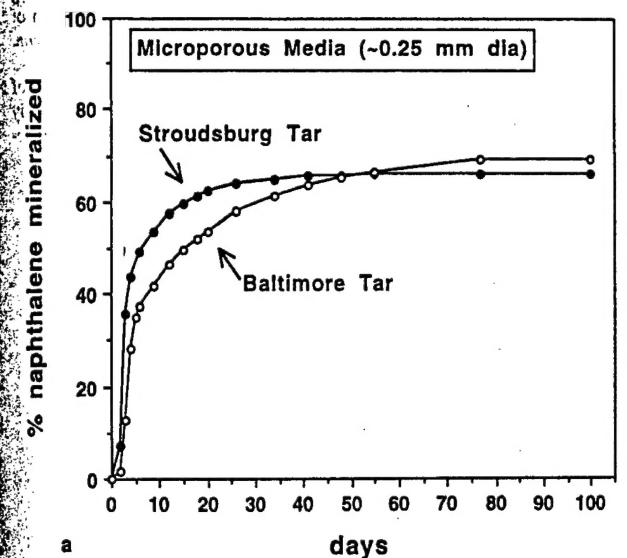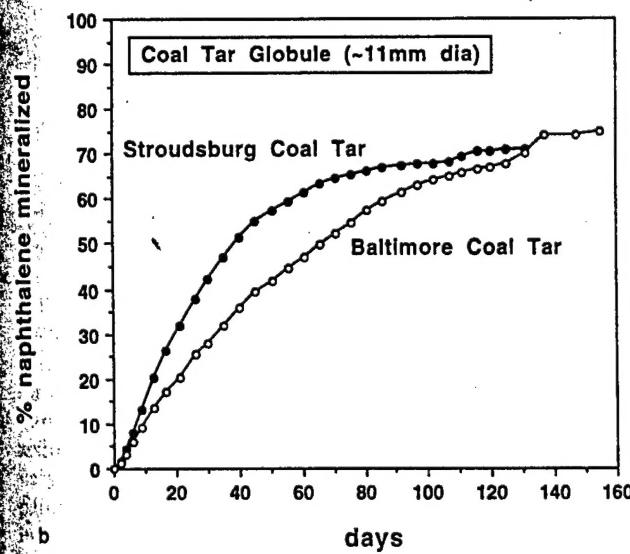
Fig. 3. Typical naphthalene mineralization profiles. (a) System with coal tar imbibed in 0.25-mm-diameter microporous silica beads. Mineralization in this system is biokinetic limited (Damkohler number much less than unity). (b) System with a 0.7-mL, ~11-mm-diameter coal tar globule. Mineralization in this system is mass transfer limited (Damkohler number larger than unity). Reprinted with permission from *Environ. Sci. Technol.* [15]. © 1996, American Chemical Society.

greater than unity suggest that the initial rate of naphthalene biodegradation is limited by the mass transfer rates.

CONCLUSION

This study shows that a very large fraction of naphthalene may be depleted from an accessible coal tar–NAPL by microorganisms in bioslurry treatment systems. It should be understood, however, that the coal tars used in this study were free-flowing tars and that the biodegradation of PAHs from more viscous or solid tars may be significantly different from that in the observed in this study.

The rate of biodegradation of naphthalene from coal tar in slurry systems can be controlled either by the intrinsic biokinetics of the microorganisms, as with coal tar imbibed in small-diameter microporous silica media, or by physicochemical factors, as with mass transfer from the larger coal tar globules. The resulting mineralization patterns may be significantly different depending on the rate limiting phenomena. Despite the complexity of the composition of coal tars and the compositional variations of coal tars from two different sources, the results obtained were consistent for both the coal tars.

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REGULATION OF MICROBIAL PHENANTHRENE MINERALIZATION IN SEDIMENT SAMPLES BY SORBENT–SORBATE CONTACT TIME, INOCULA AND GAMMA IRRADIATION-INDUCED STERILIZATION ARTIFACTS

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Abstract—Time-dependent diffusion and/or sorption reactions were proposed as a mechanism for protecting polycyclic aromatic hydrocarbons (PAHs) in surface and subsurface sediments from a coal tar waste-contaminated field site. ^{14}C-labeled phenanthrene was aged in both subsurface sand and organic matter-rich seep sediments that had previously been sterilized by gamma irradiation. After aging periods ranging from 0 to 28 d, the sediments were dispensed to replicate vials and inoculated with site-derived phenanthrene-degrading microorganisms (with and without previous enrichment on phenanthrene), and cumulative ^{14}CO$_2$ production was measured. When pure culture and mixed inocula originated from the seep sediments, phenanthrene mineralization from sand sediment samples was retarded with longer aging periods. However, when a mixed inoculum originating from the sand sediments was tested, aging of the phenanthrene had only a slight or no effect on its rate or extent of mineralization. Thus, the susceptibility of phenanthrene to biodegradation varied with the source of the microbial inocula. When gamma-irradiated seep sediments were the sorbent, all mineralization of ^{14}C phenanthrene was eliminated. Several hypotheses were tested for explaining this sorbent-dependent inhibition of phenanthrene metabolism. Gamma radiation-induced changes in the sorptive properties of the seep sediment seemed to be the cause.

Keywords—Phenanthrene   Biodegradation   Sorption   Gamma irradiation   Sediment

INTRODUCTION

Reactions between hydrophobic organic contaminant compounds and soil, organic matter-rich sediment, or organic matter-poor aquifer matrix materials have important implications for understanding and predicting the transport and fate of environmental contaminants [1–4]. The mechanisms of interaction between contaminant compounds and matrix solids can vary from formation of covalent bonds [5] to hydrophobic partitioning [6–8] to surface adsorption [9–12] to diffusion into micropores [12,13]. Regardless of the mechanism by which contaminants are sequestered in solid matrices, the net result is decreased bioavailability of the contaminant. These mechanisms have been examined from both chemical [12,14] and microbiological [11,16–19] viewpoints. A variety of investigations have concluded that both the release of model organic compounds [20,21] and their susceptibility to microbial attack [22,23] may be inversely proportional to the duration of contact between the compounds and the surrounding soil matrix. An understanding of this "aging" process can be approached using quantitative models (either discontinuous [24–26] or continuous [20]) or simply by hypothesizing that kinetically governed diffusion and hydrophobic reactions cause the partitioning of organic compounds evermore deeply in the microporous inorganic and organic structure of the matrix materials.

One of the most important implications of chemical aging processes in soil and other natural materials is the possibility that the sequestered toxic chemicals are inaccessible to mi-

crobiological attack. Analytical methods for measuring the aged organic chemical pollutants in environmental samples routinely use solvent extraction techniques, while biodegradation activity is routinely determined by following ^{14}CO$_2$ production from ^{14}C-labeled compounds freshly added to the environmental samples. A problem arises when comparing the results from the two methods because more accessible, freshly added ^{14}C-labeled hydrophobic compounds such as polycyclic aromatic hydrocarbons (PAHs) may behave differently from unlabeled PAHs whose matrix residence times are longer yet whose presence may be revealed by solvent extraction. As part of an ongoing program to discern the environmental factors and biogeochemical mechanisms governing fate of coal tar-derived organic environmental pollutants at a field study site, we examined how sorption and related reactions influence the susceptibility of phenanthrene to microbial metabolism. In particular, we set out to examine limits of the "aging–bioavailability hypothesis" [22,27] by testing key factors that may affect the results of the ^{14}C-mineralization experiments. A companion article [27] focuses on the fate of naphthalene at the same coal tar-contaminated field study site.

MATERIALS AND METHODS

Field site, sediments and bacteria

Spatial (Fig. 1), hydrogeologic, and chemical characteristics of the field study site have been described in detail elsewhere [27–30]. Sandy, uncontaminated subsurface sediment (organic matter ≈1% [20,31]) from near the source area of the field study site (Fig. 1; ~2.0–2.5 m depth) and organic matter-rich surface sediment (organic matter ≈13% [20,31]) from the contaminated "seep area" (~10–20 cm depth) were aseptically sampled and stored at 4°C. No organic, coal tar phase is in